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Handbook of Symbiotic Cyanobacteria

Edited by

Amar Nath Rai

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CRC Handbook of Symbiotic Cyanobacteria

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PREFACE

The idea for a handbook of symbiotic cyanobacteria originated during my discussions with Dr. B. Bergman and Dr. P. Lindblad while I was a visitor to the Department of Physiological Botany at Uppsala. We felt that there is a conspicuous lack of a reference source for those working on cyanobacterial symbioses, since the literature is scattered in various journals and review articles. The last 10 years have seen considerable advancement in research on cyanobacterial symbioses and their potential exploitation. In this book, I have attempted to compile the information available so far. The book consists of chapters on cyanobacterial symbioses with fungi (lichens), bryophytes, pteridophytes *(Azolla),* gymnosperms (cycads), and angiosperms *(Gunnera).* In addition, there is an introductory chapter, a chapter on artificial symbioses, and a chapter on general methods, each written by an expert in that field.

I wish to thank all those who have helped me in bringing out this book. They include all the contributors, Amy Skallerup and Sandy Pearlman of CRC Press, and my colleagues, students, and office staff at the Department of Biochemistry, NEHU, Shillong. In the preparation of this volume, the granting of copyright permissions by various publishers has been a great help, and I thank them for their liberal approach in this matter. I give a vote of special thanks to my father Shri Abhai Narain Rai, my wife Urmila Rai, and my friend Mr. David Nongrum who, in more than one way, have helped me devote the necessary time for preparation of this book.

I hope the readers find the book interesting and helpful. I shall gratefully welcome suggestions for improvement.

A. N. Rai
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THE EDITOR

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Dr. Rai is the recipient of the Principal's Gold Medal (1974) and B.H.U. Medal (1976). He was elected Fellow of the Linnean Society of London and a member of Institute of Biology, London, in 1982, and was awarded the National Scholarship for Studies Abroad by the Government of India in 1977. He has received research grants from the Department of Science and Technology, the Indian National Science Academy, and the Swedish Agency for Research Cooperation with Developing Countries.

Dr. Rai has presented over 20 papers at international conferences and has published 50 research papers. His current major research interest is in the field of cyanobacterial nitrogen metabolism, in particular nitrogen metabolism in symbiotic cyanobacteria. He is on the editorial board of the *International Journal of Lichen Physiology and Biochemistry*.

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Chapter 1

CYANOBACTERIA IN SYMBIOSIS

Amar N. Rai

THE CYANOBACTERIA

Cyanobacteria (blue-green algae) are photosynthetic prokaryotes with an oxygenic photosynthesis. Their characteristic blue-green color is due to the presence of phycobiliproteins (phycocyanin and phycoerythrin) and chlorophyll *a*. Their morphology ranges from unicellular to branched filamentous with a maximum of three cell-types: heterocysts, vegetative cells, and akinetes. In addition to having oxygenic photosynthesis, many cyanobacteria are also diazotrophic. In such cases the two processes are separated, either spatially (nitrogen fixation in heterocysts and photosynthesis in vegetative cells) or temporally (alternate cycles of photosynthesis and nitrogen fixation), to avoid deleterious effects of high oxygen concentration on nitrogenase.

Reproduction in cyanobacteria is generally by fission, budding, trichome breakage, hormogonia formation, or akinete germination. Conjugation, transformation, and transduction have also been reported. For detailed information about free-living cyanobacteria, a number of monographs are available.[1-3]

Cyanobacteria are truly ubiquitous, being distributed throughout the world under widely varying conditions. Only those which occur in symbioses are addressed in this chapter. Most organisms show structural, physiological, biochemical, and genetic adaptations to their environment. Cyanobacteria are no exception. Indeed, symbiosis as a habitat exerts considerable influence on cyanobacterial metabolism (see Chapters 1 through 7).

CYANOBACTERIAL SYMBIOSES

Elaborate discussion on the concept of symbiosis is found in works by Scott,[4] Ahmadjian and Paracer,[5] and Smith and Douglas.[6] For the purpose of this book, symbiosis means the permanant living together of two dissimilar organisms involving exchange of metabolites between the symbionts.

Cyanobacteria form symbiotic associations with plants, animals (marine sponges and echiruoid worms), nonphotosynthetic protists belonging to the group Glaucophyta, and bacteria.[6] Cyanobacteria also occur in hollow shafts of hairs of polar bears.[7,8] Among plants, cyanobacteria form symbiotic associations with algae (diatoms), fungi (lichens), bryophytes (liverworts, hornworts, and mosses), pteridophytes *(Azolla),* gymnosperms (cycads), and angiosperms *(Gunnera).* Cyanobacterial-algal symbioses, together with the cyanobacterial symbioses with animals, glaucophytes, and bacteria, are briefly addressed in this chapter. The other symbioses are discussed in detail in the following chapters.

SYMBIOTIC CYANOBACTERIA

IN ASSOCIATION WITH PLANTS

The filamentous heterocystous cyanobacterium *Richelia intracellularis* occurs in the marine pennate diatom *Rhizosolenia.* The cyanobiont* resembles *Calothrix* with a basal heterocyst. Not all *Rhizosolenia* have the cyanobiont, but those which have them may contain several filaments per cell. Some filaments have heterocysts at either end.[9] Although the diatom retains its pigments, the cyanobiont is reported to be responsible for most of the

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* The term cyanobiont has been used throughout this book to refer to the cyanobacterial partner in the symbiosis.

TABLE 1
Cyanobionts of Lichens

Unicellular forms
  Gloeocapsa
  Gloeothece
  Synechocystis
  Hyella
Filamentous heterocystous forms
  Calothrix
  Nostoc
  Scytonema
Heterocystous branched-filamentous forms
  Fischerella

Note: Grouped according to cyanobacterial classification proposed by Rippka et al.[16]

TABLE 2
Cyanobionts of Plant Symbioses Other Than Lichens

Symbiosis	Cyanobiont
Rhizosolenia (diatom)	*Richelia intracellularis*[a]
Bryophytes	*Nostoc*
Azolla	*Anabaena*[b]
Cycads	*Nostoc*
Gunnera	*Nostoc*

[a] According to criteria of Rippka et al., this should be classified as *Calothrix*.[16]

[b] According to criteria of Rippka et al., this should be classified as *Nostoc*.[16]

CO_2 fixation in the association.[10] However, the major contribution of the cyanobiont may be N_2 fixation and the transfer of fixed N to the diatom. *Rhizosolenia styliformis* is often abundant in the photic zone of the North Pacific; N_2 fixation by the cyanobiont may be of considerable significance in such nitrogen-poor oceanic waters.[10-12] The cyanobiont has not been isolated in culture and its mode of entry as well as the details of the physiology and biochemistry of this symbiosis are relatively little understood. *Richelia intracellularis* filaments have also been observed in the diatom *Hemiaulus membranaceus*, but the cyanobiont filaments are thought to persist in the diatom cell for only a few days.[6]

Inclusion bodies resembling thin-walled unicellular cyanobacteria, with thylakoids running at right angles to the cell wall, occur in the diatom *Rhopalodia*.[13] The cyanobiont fixes N_2 in the association.[14] The cyanobiont has not been isolated in culture and its precise taxonomic characterization remains to be done.

In lichens, several cyanobacterial genera (unicellular and heterocystous filamentous) have been reported,[15] but many are as yet unconfirmed. Also, some may have to be regrouped according to the new cyanobacterial classification proposed.[16] The cyanobacterial genera which occur in lichen symbioses with certainty are shown in Table 1. In all other plant symbioses, the cyanobiont is a heterocystous filamentous cyanobacterium belonging to the genus *Nostoc* or *Anabaena* (Table 2). Further details of these symbioses are given in Chapters 2 through 7.

IN ASSOCIATION WITH ANIMALS

Two groups of animals are reported to have cyanobacterial symbionts: echiruoid worms and marine sponges. Echiruoid worm-cyanobacterial symbioses are rather cryptic. However, occurrence of cyanobionts in the subepidermal connective tissues of two species *(Ikedosoma gogoshimense* and *Bonellia fuliginosa)* has been reported.[17] Prominant among the animal symbionts of cyanobacteria are the marine sponges; 38 genera belonging to the groups Calcarea and Desmospongia are known to have cyanobionts.[6,18] The cyanobionts belong to the genus *Aphanocapsa* (unicellular) or *Phormidium* (filamentous nonheterocystous). They occur, intracellularly or intercellularly, throughout the sponge tissue or in superficial tissues only. The incidence of symbiosis is very common in sponges occurring in the tropical marine environment and it has a fairly wide geographical distribution.[6,19] The distribution of cyanobionts in sponges is restricted to those occurring in the photic zone.

The role of cyanobacteria in such symbioses may be threefold:

1. Cyanobionts may protect the host sponge from damage by high light intensity.[20]
2. The cyanobiont is photosynthetically active and transfers 5 to 12% of the fixed C (probably as glycerol) to the host sponge.[19] This transfer of fixed C has been shown to be important for the host as evidenced by the fact that *Verongia aerophoba* grows faster in light than in darkness.[21]
3. In the case of at least two sponges, *Theonella swinhoei* and *Siphonochalina tabernacula,* the cyanobiont has been shown to fix N_2.[22] As in the case of diatoms, this may be an important source of nitrogen for the sponge in the N-poor marine environment.

Although the precise mechanism of cyanobiont transmission is still not known, cyanobionts have been observed in oocytes and nurse cells of several sponges.[23] This may suggest a direct transmission of cyanobiont in these associations.[6]

IN ASSOCIATION WITH BACTERIA

In addition to the cyanobacterial symbioses with various eukaryotes, there are also instances of cyanobacterial symbioses with prokaryotes. An example of this is the cyanobacterial associations with certain nonphotosynthetic bacteria in which the cyanobiont is considered to be the host.[6,24] Nonphotosynthetic bacteria occur in mucilagenous sheaths of the cyanobiont. The bacterial symbionts are often concentrated around the heterocysts or the heterocysts-vegetative cell junctions. Exchange of metabolites between the symbionts, to the mutual advantage of both, has been suggested.[24] In one case *(Pleurocapsa minor),* bacterial symbionts have been found to be located intracellularly.[25]

IN ASSOCIATION WITH NONPHOTOSYNTHETIC PROTISTS

Many nonphotosynthetic protists contain blue-green inclusion bodies in their cells. These protists include the ameba *Paulinella chromatophora* and members of the Glaucophyta.[26] The inclusion bodies are called cyanelles* and resemble unicellular cyanobacteria with a very thin cell wall. No cyanelles have been isolated in culture so far, and it remains debatable whether they should be considered as cyanobionts (organisms) or as organelles. Biochemical studies on *Cyanophora paradoxa* indicate their cyanobacterial origin,[27,28] but their genome size is far smaller than those in cyanobacteria and is comparable to plastids.[29]

Both the host and the cyanelles reproduce by binary fission, although their rates of division may vary (as in *C. paradoxa*), resulting in one to eight cyanelles per host cell.[30] In contrast, there are always two cyanelles per cell in *P. chromatophora* since the division of the cyanelles and the host is synchronous and closely coupled. Cyanelles are photosyn-

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* The term "cyanelles" was originally intended for all symbiotic cyanobacteria, but it is currently used specifically for unicellular cyanobacteria-like inclusion bodies in nonphotosynthetic protists discussed above.

TABLE 3
Classification of Symbiotic Cyanobacteria[a]

Section	Characteristics	Symbiotic cyanobacterial genera	Symbiosis
I	Unicellular; reproduction by binary fission	*Aphanocapsa*	Marine sponges
		Gloeocapsa, Gloeothece, Synechocystis	Lichens
		Aphanocapsa/Gloeocapsa	Hair of polar bears
		A thin-walled unicellular cyanobacterium	Diatom *(Rhopalodia)*
		Cyanelles	Glaucophytes and *Paulinella*
II	Unicellular; reproduction by multiple fission or by both multiple fission and binary fission	*Hyella*	Lichen
		Pleurocapsa minor	Bacteria
III	Filamentous; reproduction by random trichome breakage or by harmogonia formation; trichomes consist of vegetative cells only	*Phormidium*	Marine sponges
IV	Filamentous; reproduction by random trichome breakage, by hormogonia formation, or by akinete germination; trichomes with heterocysts; division in one plane	*Anabaena*	*Azolla*
		Nostoc	Lichens, bryophytes, cycads, and *Gunnera*
		Scytonema, Calothrix	Lichens
		Richelia intracellularis	Diatom *(Rhizosolenia)*
V	Filamentous; reproduction as in section IV; trichomes with heterocysts; division in more than one plane	*Fischerella*	Lichens

[a] According to criteria proposed by Rippka et al.[16]

thetically active and transfer up to 15% of the fixed C to the host, mainly as glucose.[30] Regarding capability of nitrogen fixation, only one association has been tested so far *(C. paradoxa)* and found to have no nitrogenase activity.[31] However, cyanelles in *C. paradoxa* do contain hydrogenase with properties similar to those in cyanobacteria.[32] Fd-dependent nitrate reductase and glutamate synthase, and glutamine synthetase activities have also been observed in the cyanelles.[33] It has been suggested that ammonia produced from nitrate/nitrite reduction in the cyanelles is made available to the host.[33] There is evidence to suggest that cyanelles do not respire due to lack of the terminal oxidase (cytochrome oxidase) in their respiratory chain.[31]

From the above information, it is clear that the cyanelles are heavily dependent upon the host regarding their metabolism. This is expected, since they do not have enough genetic information to be independent. This may explain the failure in isolating the cyanelles in culture. Attempts to grow the host in the absence of cyanelles have also failed. Thus, each symbiont seems to be dependent upon the other.[29,31,33-35]

UNIFYING CONCEPTS IN CYANOBACTERIAL SYMBIOSES

A compilation of various cyanobionts and their classification is given in Table 3. The general properties of cyanobionts (and the interaction between the symbionts) in various symbioses is summarized in Table 4. From these, the following trends are discernible (further details are in the chapters that follow):

1. The first striking thing is the range of organisms with which cyanobacteria form associations. The range is much wider than those of other diazotrophs which form

TABLE 4
Some Characteristics of Cyanobacterial Symbioses

	Diatoms	Lichens	Bryophytes	*Azolla*	Cycads	*Gunnera*	Sponges	Cyanelles
N_2 fixation	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Heterocyst frequency in the cyanobiont	Not known in *Richelia;* in *Rhopalodia* the cyanobiont is nonheterocystous	4—8% in bipartite lichens; 15—35% in tripartite lichens	Up to 43%	25—30%	Up to 46%	Up to 65%	Cyanobiont is nonheterocystous	Cyanobiont is nonheterocystous
Transfer of fixed N	Possibly yes	Yes; as ammonia	Yes; as ammonia	Yes; as ammonia	Yes	Yes	Possibly yes	Yes; ammonia from NO_3^-/ NO_2^- reduction
Levels of glutamine synthetase	Not known	5—10% of the normal level; synthesis of enzyme reduced	30% of the normal level; activity of enzyme affected	20% of the normal level; regulated at transcription level	Normal	—	—	—
Isolation of symbionts in culture	No	Yes	Yes	Yes but questionable	Yes	Yes	—	No
CO_2 fixation and transfer of fixed C	Both symbionts fix CO_2	Cyanobiont fixes CO_2 and transfers to mycobiont as glucose	No CO_2 fixation in cyanobiont; fixed C moves from bryophyte to cyanobiont	Both partners fix CO_2	No CO_2 fixation in cyanobiont; fixed C moves from cycad to the cyanobiont	No CO_2 fixation in cyanobiont; fixed C moves from *Gunnera* to the cyanobiont	Cyanobiont fixes CO_2 and transfers it to the host as glycerol	Cyanobiont fixes CO_2 and transfers it to the host as glucose

Note: — indicates not known.

symbioses (e.g., *Rhizobia* and *Frankia*). This, together with the fact that cyanobacteria combine oxygenic photosynthesis with N_2 fixation, indicates that cyanobacteria may be a potential candidate for use in creating artificial diazotrophic symbioses. Further details on artificial symbioses are provided in Chapter 8.

2. The range of cyanobacteria occurring in the symbioses indicates that most cyanobionts are diazotrophic. This suggests provision of fixed N as a major role of cyanobionts in these symbioses. Although the form in which fixed N moves from cyanobiont to the eukaryotic partner may vary, ammonia seems to be the major compound in the cases so far examined.
3. Heterocyst frequency, which is normally approximately 5% in free-living cyanobacteria, increases significantly in cyanobionts where the cyanobiont is in association with a photosynthetic partner. Since heterocysts are the site of nitrogen fixation in filamentous heterocystous cyanobacteria, the cyanobionts with high heterocyst frequency carry out N_2 fixation at higher rates.
4. In several symbioses, the cyanobiont has low levels of glutamine synthetase. From the cases examined so far, it has become apparant that two mechanisms are involved. In some the regulation is at transcription level, while in others it is at the activity level. Insufficient levels of glutamine synthetase in the cyanobiont results in liberation of the newly fixed ammonia (produced during N_2 fixation). The distribution of the residual glutamine synthetase in the cyanobiont is uniform between vegetative cells and heterocysts. This is in contrast to the free-living cyanobacteria where heterocysts contain twice the amount of glutamine synthetase present per vegetative cell (see Chapter 2).
5. Cyanobionts which occur in association with heterotrophic symbionts retain their photosynthetic ability, but those in association with photosynthetic symbionts fix CO_2 at low levels or not at all. In the latter case, fixed C is provided by the eukaryotic partner to the cyanobiont. Thus, with regard to fixed C transfer, the cyanobacteria symbioses fall into three categories: those symbioses where the cyanobiont transfers both fixed C and fixed N to the eukaryotic partner (e.g., bipartite lichens); those symbioses where the cyanobiont transfers only fixed N but remains self-sufficient for its fixed C requirement (e.g., tripartite lichens and *Azolla*); and those symbioses where the cyanobiont transfers fixed N but receives fixed C from the host because it is functionally nonphotosynthetic (e.g., liverworts, cycads, and *Gunnera*).

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REFERENCES

1. **Fogg, G. E., Stewart, W. D. P., Fay, P., and Walsby, A. E.,** *The Blue-Green Algae,* Academic Press, London, 1973.
2. **Carr, N. G. and Whitton, B. A.,** *The Biology of Cyanobacteria,* Blackwell Scientific, Oxford, 1982.
3. **Fay, P.,** *The Blue-Greens,* Edward Arnold, London, 1983.
4. **Scott, G. D.,** *Plant Symbioses,* Edward Arnold, London, 1969, 1.
5. **Ahmadjian, V. and Paracer, S.,** *Symbiosis,* University Press of New England, Hanover, NH, 1986, 1.
6. **Smith, D. C. and Douglas, A. E.,** *The Biology of Symbiosis,* Edward Arnold, London, 1987, 1.
7. **Lewin, R. A. and Robinson, P.,** The greening of polar bears in zoos, *Nature (London),* 278, 445, 1979.
8. **Lewin, R. A., Farnsworth, P. A., and Yamanaka, G.,** The algae of green polar bears, *Phycologia,* 20, 303, 1981.

9. **Fritsch, F. E.,** *The Structure and Reproduction of Algae,* Vol. 2, Cambridge University Press, London, 1945.
10. **Mague, T. H., Weare, N. M., and Holm-Hansen, O.,** Nitrogen fixation in the North Pacific Ocean, *Mar. Biol.,* 24, 109, 1974.
11. **Mague, T. H., Mague, F. C., and Holm-Hansen, O.,** Physiology and chemical composition of nitrogen-fixing phytoplankton in central North Pacific Ocean, *Mar. Biol.,* 41, 213, 1977.
12. **Venrick, E. L.,** The distribution and significance of *Richelia intracellularis* Schmidt in the North Pacific central gyre, *Limnol. Oceanogr.,* 19, 437, 1974.
13. **Drum, R. W. and Pankratz, S.,** Fine structure of an unusual cytoplasmic inclusion in the diatom genus *Rhopalodia, Protoplasma,* 60, 141, 1965.
14. **Floener, L. and Bothe, H.,** Nitrogen fixation in *Rhopalodia gibba,* a diatom containing blue-greenish inclusions symbiotically, in *Endocytobiology, Endosymbiosis and Cell Biology,* Vol. 1, Schwemmler, W. and Schenk, H. E. K., Eds., Walter de Gruyter, Berlin, 1980, 541.
15. **Ahmadjian, V.,** A guide to the algae occurring as lichen symbionts: isolation, culture, cultural physiology and identification, *Phycologia,* 6, 127, 1967.
16. **Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y.,** Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J. Gen. Microbiol.,* 111, 1, 1979.
17. **Kawaguti, S.,** Blue green algae in echiuroid worms, in *Aspects of the Biology of Symbiosis,* Chang, T. C., Ed., Butterworths, London, 1971, 265.
18. **Wilkinson, C. R.,** Physiology of bacterial and cyanobacterial symbionts in marine sponges, in *Endocytobiology, Endosymbiosis and Cell Biology,* Vol. 2, Schenk, H. E. K. and Schwemmler, W., Eds., Walter de Gruyter, Berlin, 1983, 993.
19. **Wilkinson, C. R.,** Net primary productivity in coral reef sponges, *Science,* 219, 410, 1983.
20. **Sara, M.,** Ultrastructural aspects of the genus *Aphanocapsa* (Cyanophyceae) and *Ircinia variabilis, Mar. Biol.,* 11, 214, 1971.
21. **Wilkinson, C. R. and Vacelet, J.,** Transplantation of marine sponges to different conditions of light and current, *J. Exp. Mar. Biol. Ecol.,* 37, 91, 1979.
22. **Wilkinson, C. R. and Fay, P.,** Nitrogen fixation in coral reef sponges with some symbiotic cyanobacteria, *Nature (London),* 279, 527, 1979.
23. **Fell, P. E.,** Porifera, in *Reproductive Biology of Invertebrates. I. Oogenesis, Oviposition and Oosorption,* Adiyodi, K. G. and Adiyodi, R. G., Eds., John Wiley & Sons, Chichester, England, 1983, 1.
24. **Paerl, H. W.,** Interactions with bacteria, in *The Biology of Cyanobacteria,* Carr, N. G. and Whitton, B. A., Eds., Blackwell Scientific, Oxford, 1982, 441.
25. **Wujek, D. E.,** Intracellular bacteria in the blue-green alga *Pleurocapsa minor, Trans. Am. Microsc. Soc.,* 165, 908, 1979.
26. **Reisser, W.,** Endosymbiotic cyanobacteria and cyanellae, in *Cellular Interactions, Encyclopedia of Plant Physiology, New Series,* Vol. 17, Pirson, A. and Zimmermann, M. H., Eds., Springer-Verlag, Berlin, 1984, 91.
27. **Jaynes, J. M. and Vernon, L. P.,** The cyanelle of *Cyanophora paradoxa,* almost a cyanobacterial chloroplast, *Trend Biochem. Sci.,* 7, 22, 1982.
28. **Heinhorst, S. and Shively, J. M.,** Encoding of both subunits of ribulose-1,5-bisphosphate carboxylase by organelle genome of *Cyanophora paradoxa, Nature (London),* 304, 373, 1983.
29. **Herdman, M. and Stanier, R. Y.,** The cyanelle: chloroplast or endosymbiotic prokaryote?, *FEMS Microbiol. Lett.,* 1, 7, 1977.
30. **Trench, R. K., Pool, R. R., Logan, M., and Engelland, A.,** Aspects of the relationship between *Cyanophora paradoxa* (Korschikoff) and its endosymbiotic cyanalles *Cyanocyta korschikoffiana* (Hall and Claus), *Proc. R. Soc. London Ser. B,* 202, 423, 1978.
31. **Floener, L. and Bothe, H.,** Metabolic activities in *Cyanophora paradoxa* and its cyanelles. II. Photosynthesis and respiration, *Planta,* 156, 78, 1982.
32. **Bothe, H. and Floener, L.,** Physiological characterization of *Cyanophora paradoxa,* a flagellate containing cyanelles in endosymbiosis, *Z. Naturforsch.,* 33c, 981, 1978.
33. **Floener, L., Danneberg, G., and Bothe, H.,** Metabolic activities in *Cyanophora paradoxa* and its cyanelles. I. The enzymes of assimilatory nitrate reduction, *Planta,* 156, 70, 1982.
34. **Bayer, M. G. and Schenk, H. E. A.,** Biosynthesis of proteins in *Cyanophora paradoxa.* I. Protein import into the endocyanelle analysed by micro two-dimensional gel electrophoresis, *Endocytol. Cell Res.,* 3, 197, 1986.
35. **Trench, R. K.,** Physiology, biochemistry, and ultrastructure of cyanellae, in *Progress in Phycological Research,* Vol. 1, Round, F. E. and Chapman, D. J., Eds., Elsevier Biomedical, Amsterdam, 1982, 257.

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Chapter 2

CYANOBACTERIAL-FUNGAL SYMBIOSES: THE CYANOLICHENS

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INTRODUCTION

Lichens are symbiotic associations between certain fungi and photosynthetic organisms (cyanobacteria and/or algae) resulting in a stable thallus quite distinct from either of the symbionts occurring in a free-living state. They represent an integration of the heterotrophic metabolism of the fungal partner (mycobiont) and the autotrophic metabolism of the photosynthetic partner/partners (photobionts). There are approximately 15,000 to 20,000 known lichen species, of which the majority have only one photobiont (bipartite lichens); however, there are about 520 species of tripartite lichens which contain two photobionts: a green alga (phycobiont) and a cyanobacterium (cyanobiont).[1-4]

In this chapter only those lichens which contain cyanobacteria as photobionts (the cyanolichens) are discussed. For a detailed account of lichens in general, the reader is referred to the most recent monograph on this subject.[5]

THE SYMBIONTS

THE MYCOBIONT

There are approximately 13,500 species of lichen-forming fungi representing nearly 20% of all known fungal species.[4] The mycobiont is generally an ascomycete (about 40 genera). However, 10 genera and 20 species of Basidiomycetes also occur as mycobionts. Some Fungi Imperfectii and at least one phycomycetous fungus have also been reported as mycobionts.[3]

THE PHYCOBIONT

Lichens generally contain eukaryotic green algae as photobionts. Most common among the phycobionts are *Trebouxia* and *Pseudotrebouxia,* which occur in nearly 70% of lichens. Other common phycobionts are *Coccomyxa* and *Myrmecia.*

THE CYANOBIONT

About 8% of lichen species have cyanobacteria as photobionts. Within the lichen thallus, cyanobionts develop extracellularly except in one case, *Geosiphon pyriforme,* where the cell wall of the phycomycetous fungus is lysed and the *Nostoc* cells are enclosed by invagination of the plasmalemma.[6,7] In bipartite lichens, the cyanobiont occurs either throughout the thallus (Homoiomerous type lichen thallus; e.g., *Collema* sp.) or is restricted to a distinct symbiont layer in the upper cortex (Heteromerous type lichen thallus; e.g., *Peltigera canina*). In tripartite lichens, however, the cyanobiont occurs in special structures called cephalodia. Cephalodia may be external, occurring on the outer surface of the thallus (e.g., in *P. aphthosa*), or internal, occurring within the thallus (e.g., in *Lobaria pulmonaria*). Occasionally, the cyanobiont and the phycobiont both develop as discrete layers within the main thallus (e.g., in *Solorina crocea*). The cyanobiont is also termed the secondary photobiont and the phycobiont the primary photobiont in tripartite lichens.

There has been a great deal of uncertainty about the cyanobacterial genera occurring in lichen symbioses. This has been mainly due to the fact that most reports are based on only direct microscopic observations and that in relatively few cases the cyanobionts have been isolated in culture. There is indeed evidence to show that at least in some cases the morphology of the cyanobacterium may change drastically. For example, Marton and Galun[8] found that the filamentous heterocystous cyanobacterium *Scytonema* becomes unicellular and has no heterocysts in the lichen *Heppia echinulata*. When isolated and cultured, the cyanobiont reverts to its original character. This highlights the need for studying the cyanobiont under culture before making a taxonomic characterization.

Ahmadjian[9] has compiled a list of cyanobacterial genera reported in lichen symbioses. Taking this as a base and applying the cyanobacterial taxonomic criteria proposed by Rippka et al.,[10] a list of cyanobacterial genera occurring in lichen symbioses has been tabulated (see Tables 1 and 2 of the previous chapter).

Among heterocystous cyanobacteria, four genera have been shown to occur as lichen photobionts with certainty. These are

- *Calothrix* (e.g., in *Lichina* spp.,[11-13] *Calotrichopsis,*[14] *Poroscyphus,*[14] and *Placynthium nigrum*[15])
- *Nostoc* (e.g., in *Collema* spp.,[16] *Peltigera* spp.,[17-19] *Hydrothyria venosa,*[20] *Pannaria* sp.,[19] *Leptogium issatschenkii,*[21] and *Stereocaulon* sp.[22])
- *Scytonema* (e.g., in *Pannaria, Coccocarpia, Erioderma, Dictyonema;*[12,23] *Zahlbrucknerella, Lichinodium, Polychidium, Thermutis;*[14] *Lichenothrix;*[14] *Heppia;*[8] and *Lasioloma, Lopadium,* and *Stereocaulon*[12,22,23,25])
- *Fischerella* (e.g., in *Ephebe, Spilonema,* and *Stereocaulon*[12,22,25-28])

Among unicellular cyanobacteria, *Hyella* is reported to occur in *Arthopyrenia halodytes.*[29] Other unicellular cyanobacteria occurring in lichen symbioses have been grouped in three genera: *Gloeocapsa, Gloeothece,* and *Synechocystis.* In the absence of full details to enable precise classification, even this may not be entirely correct. However, it is certain that unicellular cyanobacteria do occur in lichen symbioses. Some of their reported occurrences in lichens include: *Pyrenopsidium, Phylliscum,*[12,30] *Pterygiopsis,*[9,14] *Synnalisa,*[12,30,31] *Psorotichia,*[30] *Pyrenopsis,*[30,31] *Peccania,*[31,32] *Stereocaulon,*[12,22] *Peltula, Gloeoheppia, Gonohymenia, Pyrenopsidium, Lichinella,* and *Thyrea.*[32] Bubrick and Galun[32] have isolated in culture the unicellular cyanobionts from 17 members of Lichinaceae and Heppiaceae and found a wide range of unicellular forms. Most resemble *Gloeocapsa,* but their taxonomy has not been considered in detail.[32]

DISTRIBUTION OF LICHENS

Lichens grow on a wide range of organic and inorganic substrates under widely varying conditions worldwide. They are most common in terrestrial habitats, but many species also occur in aquatic habitats (e.g., *Hydrothyrea venosa* in fresh waters and *Lichina* spp. in marine waters). Their wide distribution is mainly due to their ability to colonize relatively barren environments where other plants fail. They often form dominant vegetation in tundra and arctic areas. Lichens have been reported from such unusual habitats as tortoise shells, glass windows, and living leaves of tropical trees and ferns. Lichens are successful colonizers of desert and rocky habitats. They are abundant in temperate and tropical rain forests, rocky seashores, and tree barks in humid tropics. Lichens are very sensitive to environmental pollution and are therefore often absent from city and industrial habitats.

MORPHOLOGY

Lichen thalli exhibit great complexity of form and color. Most lichens are gray or brown when dry, but the color of the photobiont is distinctive in wet thalli. Many species are brightly colored by incrustations of lichen pigments in the cortex. Green and yellow tints are more common. Based on growth forms, lichens can be grouped into three categories: crustose, foliose, and fruticose.

Crustose lichens lack a lower cortex and are tightly attached to the substratum by medullary hyphae. Crustose lichens are most abundant and consist of little scales (areoles). Lichens with very small areoles are often homoiomerous. Bigger areoles start to show signs of heteromerous composition. Some crustose lichens consist of small lobes (squamules). Examples of such squamulose cyanolichens are *Heppia, Lecidea,* and *Placynthium.* These thalli consist of small scales, whose margins separate from the substrate on one side and bend upwards, arranged in a rosette.

The foliose lichen thallus is composed of heteromerous flattened lobes which are dorsiventral in structure. They are generally loosely attached to the substrate by rhizines. Their habitat, anatomy, and mode of attachment varies greatly. Some examples of foliose lichens are *Peltigera* spp., *Lobaria* spp., and *Stereocaulon* spp.

Fruticose lichen thalli are round or flat, often highly branched, and vertical or hanging down from their substrate to which they are attached by a holdfast. Examples of cyanolichens in this category are *Sticta filix, Pilophorus strumaticus,* and *Stereocaulon alpinum.*

Some cyanolichens do not form a well-organized thallus (e.g., *Collema*). The thallus structure is generally homoiomerous with fungal hyphae growing inside the thick gelatinous sheaths of the cyanobionts.

THALLUS STRUCTURE (ANATOMY)

The primitive lichen thalli consist of a loose fungal mycelium enclosing groups of algae and spreading over the substrate. Others resemble a gelatinous algal colony penetrated and interwoven by fungal hyphae (e.g., some *Collema* spp. and *Leptogium* spp.). Such thalli are termed homoiomerous.

Most lichens are structurally more complex. The cyanobionts are restricted to a particular layer in the thallus or occur in cephalodia. In addition to the photobiont layer, there is at least one more defined layer (medulla) which is devoid of photobionts. Such thalli are termed heteromerous.

The photobiont layer is generally just below the upper surface of the lichen. The photobiont is extracellular but in close contact with the mycobiont hyphae. In some lichens haustoria penetrate the photobiont cells (e.g., in *Dictyonema*). The thickness of the cyanobiont layer varies. The region immediately above the cyanobiont layer is called the upper cortex and the region immediately below the cyanobiont layer is termed the medulla. Below the medullary region is the lower cortex. The medulla consists of loosely interwoven mycobiont hyphae, has great water holding capacity, and serves as a region of food storage.

Homoiomerous lichen thalli are attached to the substrate by the basal hyphae. In heteromerous lichens, fungal hyphae originating from the lower cortex form rhizines. In the Peltigeraceae and Lobariaceae, young rhizines consist of loosely associated hyphae which later become closely connected. Mature rhizines spread at their tips and become brush-like. In *Leptogium,* rhizines are produced by tufts of individual hyphae not connected with each other. It is unknown whether rhizines play a role in transport of metabolites from substrate to thallus and, if they do so, to what extent.

How the cyanobiont is trapped and transported from older to younger regions of the thallus for continued thallus synthesis and growth is not known in most cyanolichens. However, in *Placynthium nigrum,* an interesting mechanism has been found.[33] The thallus squamules, which contain the cyanobiont, are surrounded by a hair-like prothallus. Hormogonia, produced by the cyanobiont, are washed off by the water from the margin of the scales. These come to rest on the prothallus where they are enclosed by the fungal hyphae followed by formation of new squamules on the prothallus. These continue to grow and finally reach the older squamules, forming a continuous system of scales.

Several lichen thalli have been found to contain aeration pores between the cortical hyphae.[34] Their precise role is not entirely certain. In *Sticta,* these aeration pores are called cyphellae. Similar openings called tubercles occur on the lower side of *Nephroma* thalli. Aeration pores in certain other species (e.g., in *Pseudocephallaria*) are called pseudocyphallae. The distinction between cyphellae and pseudocyphellae is rather arbitrary. In some lichens (e.g., *Placopsis*), when the isidia break off they leave little warts on the thallus with a central opening filled by loose hyphae. These openings serve as aerating organs.[33]

In tripartite lichens, the photobiont layer is always occupied by the phycobiont (the primary photobiont). The position of the cyanobiont (the secondary photobiont) varies. In some lichens (e.g., *Solorina crocea*), the cyanobiont *(Nostoc)* occurs beneath the layer of the primary photobiont *(Coccomyxa).* In the fruticose lichen *Compsocladium,* cyanobiont filaments lie singly in the medulla while the primary photobiont occupies the subcortical photobiont layer. However, in most tripartite lichens, the cyanobiont lies in special delimited, swollen structures called cephalodia. Cephalodia are either sited within the thallus, in the medulla (e.g., *Lobaria* and *Sticta*), or on the thallus surface (e.g., *Peltigera aphthosa, Placopsis, Pilophorus, Stereocaulon,* etc.). The shape of external cephalodia varies considerably, being round *(Pilophorus, Stereocaulon),* disc-shaped *(Placopsis, Peltigera aphthosa),* or branched and bushy. A cephalodial cyanobiont is often specific for a cyanolichen (e.g., *Nostoc* in *Lobaria* and *Sticta*). However, in some cases, different cyanobionts occur even in adjoining cephalodia on the same thallus (e.g., *Gloeocapsa* and *Fischerella* in cephalodia of *Lecidea* and *Pelobotrya*).[35] Cephalodia formation is also used as taxonomic criterion. All genera belonging to Stereocaulaceae have cephalodia. In addition, many genera of Calciaceae, Lecideaceae, and Peltigeraceae form cephalodia. Other genera which regularly form cephalodia are *Placopsis* and *Psoroma.*

Development of cephalodia in *Peltigera* starts by entrapment of the cyanobiont by hairs on the surface of the thallus[36] followed by involvement of more mycobiont hyphae. The cortex of the thallus, immediately below the cephalodium, disintegrates and establishes a direct contact with medullary hyphae. The phycobiont layer immediately below the cephalodium is thus disrupted. Internal cephalodia are formed in a similar manner starting with entrapment of cyanobiont by cortical hyphae.[36-38] The cyanobiont enmeshed by a thick layer of mycobiont is pressed into the thallus where the cephalodium eventually develops. The cyanobiont usually penetrates via the lower surface, but occasionally when it enters via the upper surface, the cephalodium lies above the phycobiont layer which is pushed deeper into the medulla.

Although the cyanobiont has some influence on the lichen thallus structure, in most lichens mycobiont is the dominant partner and has a major influence on the lichen thallus structure.[33] This is evident from the fact that different fungi forming lichens with the same cyanobiont have differently organized thalli. For example, some genera belonging to the Lichinaceae or Imperfectii groups all contain *Scytonema* as the cyanobiont, but the lichen structure varies. In *Lichenothrix* and *Thermutis,* the appearance of the photobiont is unchanged by the fungal hyphae.[14,24] In *Thermutis* the fungal hyphae grow inside the cyanobiont sheath, while in *Lichenothrix* only haustoria penetrate the sheath and other hyphae remain outside the sheath. In *Zahlbrucknerella* and *Lichinodium,* the cyanobiont is so closely enveloped by the mycobiont hyphae that the pseudobranching in the cyanobiont is masked. In *Polychidium,* the fungus is so dominant that the cyanobiont is invisible from the outside. Similarly, there are a large number of lichens, with varying structure and belonging to different groups, all have *Nostoc* as cyanobiont. Interesting chimeroid associations have been reported in the genera *Peltigera* and *Sticta.*[39,40]

CLASSIFICATION

Using Poelt's classification of lichens,[41] the cyanolichens may be grouped as follows:

Ascolichens	
Pleosporaceae	*Arthopyrenia*
Sphaerophoraceae	*Thysanophoron, Sphaerophorus*
Thelotremataceae	*Petractis*
Caliciaceae	*Chaenotheca*
Lichinaceae	*Anema, Calotrichopsis, Ephebe, Gloeoheppia, Gonohymenia, Lemmopsis, Lempholemma, Lichina, Lichinella, Lichinodium, Phylliscidium, Phylliscum, Phyllisciella, Peccania, Poroscyphus, Psorotichia, Pterygiopsis, Pyrenopsidium, Pyrenopsis, Rechingera, Synalissa, Thermutis, Thyrea, Zahlbrucknerella*
Heppiaceae	*Heppia, Peltula*
Placynthiaceae	*Koerberia, Massalongia, Placynthium, Polychidium, Psoroma, Psoromaria, Steinera, Vestergrenopsis*
Peltigeraceae	*Hertella, Hydrothyrea, Peltigera, Solorina, Dendriscocaulon*
Nephromaceae	*Nephroma*
Lobariaceae	*Lobaria, Pseudocephellaria, Sticta*
Collamataceae	*Collema, Homothecium, Leciophysma, Leightoniella, Leptogium, Physma, Ramalodium*
Pannariaceae	*Degelia, Erioderma, Pannaria, Parmeliella*
Coccocarpiaceae	*Coccocarpia, Spilonema*
Arctomiaceae	*Arctomia*
Lecideaceae	*Lecidea, Lasioloma, Lopadium*
Stereocaulaceae	*Agropsis, Compsocladium, Pilophorus, Stereocaulon*
Pertusariaceae	*Coccotrema, Lepolichen*
Trapeliaceae	*Placopsis* (includes *Aspiciliopsis*)
Basidiolichens	
Dictyomataceae	*Dictyonema* (includes *Cora* and *Corella*)
Clavariaceae	*Multiclavula*
Lichen Imperfectii	*Lichenothrix* (includes *Pyrenothrix)*

REPRODUCTION

Reproduction in lichens is mainly by vegetative means. Sexual reproduction occurs in the mycobiont, but in the cyanobiont sexual reproduction in symbiosis is not yet reported. The cyanobiont reproduces through vegetative means only.

Vegetative structures (propagules) develop frequently and help in dispersal and propagation of lichens. Such propagules include phyllidia, isidia, soredia, and hormocystangia.

Phyllidia (also called "isidia squamiformia", lobulae, or lobules) are foliose, flattened, dorsiventral growths of the upper cortex or thallus margin.[42] They usually have a constricted base and fall away as diaspores. Phyllidia have been reported in such cyanolichens as *Collema, Peltigera,* and *Sticta.*

Hormocystangia are swollen structures at the ends or margins of the lobes in some species of *Lempholemma.*[43,44] They consist of lichenized and/or unlichenized hormocysts. These are short cyanobiont *(Nostoc)* filaments enclosed by a thick gelatinous sheath penetrated by fungal hyphae. Lichenized hormocysts are released on hormocystangia decay and help in propagation. Nonlichenized hormocysts help in dispersal and propagation of the cyanobiont only.

Isidia are small protuberances of the thallus and consist of cyanobiont and medullary tissue covered by a cortex. Their forms vary, being globular, cylindrical, coralloid, or scale-shaped. They range from 0.01 to 0.03 mm in diameter and 0.5 to 3 mm in height. Isidia formation occurs in crustose, foliose, and fruticose lichens. Isidia are formed by 25 to 30% of foliose lichen species, but are rarer in crustose forms.

Soredia are minute diaspores consisting of cyanobiont closely enveloped by mycobiont hyphae. Individual soredia are 25 to 100 μm in diameter, they often adhere together, forming powdery or granular masses which are water-repellent. Soredia occur in structures called sorelia that look like powdery breaks in the thallus cortex. Soredia differ from isidia in being devoid of cortex and, depending upon their shape and location, they are grouped as maculiform, rimiform (fissure-shaped), convex-globular, maniciform (cuff-shaped), labriform (lip-shaped), marginal, and parietal.[45-47]

Water, wind, insects, birds, and animals have been suggested as means of diaspore dispersal in lichens.[48-59]

ULTRASTRUCTURE

Ultrastructural studies on lichens were initiated during the 1960s.[60,61] For full details on ultrastructure of lichens and their symbionts, the reader is referred to various reviews on lichen ultrastructure[34,62-66] and the recent monograph on lichens.[5] Here the discussion is restricted to ultrastructural aspects of the cyanobiont and the contacts between cyanobiont and mycobiont in cyanolichens. The cyanobiont has no direct contact with the phycobiont in tripartite lichens, where both occur together as photobionts.

Most of the ultrastructural studies have been done on ascolichens containing *Nostoc* or *Scytonema* cyanobionts, with the exception of *Gonohymenia* spp. which contain *Gloeocapsa*.[67] So far, only one study has been conducted on ultrastructure of basidiolichens; that is on *Cora pavona (Dictyonema*?).[68]

In general, the cyanobiont ultrastructure is comparable to the free-living forms having usual cell layers, polyhedral bodies (carboxysomes), polyphosphate bodies, cyanophycin granules, phycobiliproteins, plastoglobules, etc. There are, however, some noticeable differences.

A comparison of the *Nostoc* isolate from *P. canina* with the cyanobiont in thallus indicated that in symbiosis there is an increase in vegetative cell size (from 2.5 to 4.5 μm diameter) and thylakoid numbers. The arrangement of thylakoids in the cyanobiont has been noted to be reticular and spread throughout the cytoplasm. Heterocyst frequencies in the isolate and the cyanobiont are comparable, except that in the thallus margins the cyanobiont contains fewer heterocysts. Akinetes are rarely seen in the lichen cyanobionts, but have been observed in *P. canina*.[69,70]

Microtubule-like structures have been observed in *Nostoc* cyanobionts of *Collema tenax* and *Leptogium cyanescens*;[71,72] however, their function is unknown.

Polyglucoside bodies (glycogen granules) have been reported to be absent in some cyanobionts.[67,71] However, in several other cyanobionts, including *Nostoc* (in *Peltigera* spp., *Hydrothyrea venosa, Collema tenax,* and *L. lichenoides*) and *Fischerella* (in *Stereocaulon paschale*), polyglucoside granules are reported to be abundant.[69,73-76] These granules have been shown to be dynamic glycogen reserves which are highly mobile and affected by the environmental conditions and metabolic state of the thallus.[75,76] Therefore, they may or may not be observed in the lichen cyanobiont depending on the season and time of the lichen collected.[76]

An increase in lipid granules (plastoglobuli or osmiophilic granules) has also been noted in the lichen cyanobionts.[61,67,70,74,77] In the cyanobionts of *Peltigera* spp. *(Nostoc)* these globuli are in singles with a diameter of 60 to 100 nm, while in the *Nostoc* cyanobiont of *L. hildenbrandii* they are in groups of three to eight with a diameter of about 50 nm. *Gloeocapsa* cyanobiont in *Gonohymenia* spp. also contains such globuli, although they are less regular in shape.[67]

Phycobilisomes and phycobiliproteins have been noted in cyanobionts of *Peltigera* spp.,[69,78-80] but the composition varies. For example, phycocyanin was found to be absent in the *P. aphthosa* cyanobiont.[78]

Certain differences in the cell wall structure of the cyanobiont in various lichens have been noted. *Nostoc* in *P. polydactyla* has only an inner wall and a fibriller cell sheath,[61] while *Nostoc* in *L. hildenbrandii, P. canina,* and *P. rufescens,* as well as *Gloeocapsa* in *Gonohymenia* spp., shows a more detailed wall consisting of four small zones of alternate contrast.[67,81] In addition to these four layers, there is a fibriller outer sheath. In *Peltigera, Nostoc* has tightly packed fibers forming a 100-nm thick sheath, while in *Nostoc* of *L. hildenbrandii* the sheath is up to 1000 nm wide.[81] Vesicular structures, formed by proliferation of the membrane-like part of the cell wall, have been noted in *Calothrix* cyanobiont of *Lichina pygmea*[82] and in the *Nostoc* cyanobiont of *P. canina, Collema lingerinum,* and *P. aphthosa.*[83,84] Although their role is uncertain, suggestions have been made that they are transfer structures.[82]

Detailed ultrastructural studies have been conducted on the types of contacts which develop in lichen symbioses between cyanobiont and the mycobiont. In most lichens there is close physical contact between the two partners without the mycobiont hyphae penetrating the cyanobiont cell wall. This includes close cell-cell contact by thinning of cell walls and thin-walled mycobiont hyphae or protuberances entering the gelatinous sheath of the cyanobiont.[65,70] However, haustoria have been observed in several lichen species. These are specialized peg-like or plug-like projections of mycobiont which penetrate the cyanobiont cell wall (intracellular haustoria) or which may be intraparietal (intramembranous/intrawall haustoria). Haustoria have been observed in *Peltigera* spp.,[70] *Lecidea* sp.,[85] *Gonohymenia,*[67] *Lichina pygmea, C. pavonia (Dictyonema),*[68] etc. Haustoria are rare in lichens containing *Nostoc,* but are more common in lichen with *Scytonema* or *Calothrix* cyanobionts. Intracellular haustoria are more common in primitive lichens.[2,87] Haustoria contain higher numbers of mitochondria[88] and may be involved in nutrient exchange/transfer. However, this is not an obligatory phenomenon for the mutual relationship between lichen symbionts.[89,90]

RECOGNITION

From the foregoing discussion, it is evident that in lichen symbioses there is a significant degree of selectivity regarding the cyanobiont with which the mycobiont establishes a lichen symbiosis. Such selectivity may involve some type of recognition mechanism to ensure association with the ''right'' cyanobiont. Recent studies have indicated that some such mechanism may indeed be used by the mycobiont to select the right cyanobiont during initial stages of the establishment of a lichen symbiosis.

Lectins, which are implicated in recognition events during establishment of *Rhizobium*-legume symbioses,[91,92] have been reported in several cyanolichens including *P. canina,*[93-95] *P. polydactyla,*[94,96] *P. aphthosa,*[97,98] *P. horizontalis,*[99] *P. rufescens,*[97] *P. leucophlebia,*[97] *P. praetextata,*[100] *Nephroma arcticum,*[93] *P. malacea,*[93] *Placynthium asperellum,*[93] *Psoroma hypnorum,*[93] and *Stereocaulon vesuvianum.*[93] From the studies on cyanolichens conducted so far, there is growing evidence to suggest that lectins may mediate selective events in, at least, *Nostoc*-containing lichens.[94,95,99] The evidence is as follows:

1. Lockhart et al.[94] found that in *Peltigera canina* and *P. polydactyla,* lectins of mycobiont origin bound strongly to the cultured cyanobiont. They suggested that such lectins may be involved in initial stages of establishment of nitrogen-fixing symbioses. Subsequently, lectins were reported in *Azolla-Anabaena* associations (see also Chapter 4).[101,102]
2. Petit[99] and Petit et al.[95] have shown that the lectins produced by *P. canina* and *P. horizontalis* mycobionts bind to the cultured *Nostoc* cyanobiont from *Peltigera* spp., and have suggested their involvement in recognition and initial interaction between compatible lichen symbionts. These lectins do not bind to the freshly isolated cyanobionts.

3. Lallemant and Savoy,[100] using *P. canina* lectin conjugated with fluoroceine isothiocyanate (FITC), have visualized lectin-binding sites in *P. praetextata*. They indicated that only 20% of cyanobiont trichomes show FITC labeling because the other 80% had their lectin binding sites occupied by endogenous lectins. They suggested that lectins play a major role in the recognition process between the symbiotic partners.

Dick and Stewart[103] have reported occurrence of fimbriae in lichen cyanobionts of *P. canina* and suggested that these may be involved in motility and specificity in cyanobionts.

CO_2 FIXATION AND TRANSFER OF FIXED CARBON

CO_2 FIXATION

In all lichens studied thus far, the cyanobiont is functionally photosynthetic, fixes CO_2, and transfers fixed C to the mycobiont. Lichen metabolism is highly adaptable to seasonal variations. The ecophysiological aspects of photosynthesis; gaseous exchange and respiration; and the impacts of moisture, temperature, and light on these processes have been covered in detail in a recent monograph on this subject.[104]

The mechanism of CO_2 fixation in cyanobionts studied is similar to that in free-living cyanobacteria. ^{14}C-labeling experiments in *P. polydactyla*,[105] *C. pavonia (Dictyonema)*,[106] and *P. canina*[79] have shown that phosphoglyceric acid is the most highly labeled initial product after 30 s of exposure; with time, the proportion of label in phosphoglyceric acid declined and labeling in other compounds increased. This indicated that the primary carboxylation reaction is the ribulose-1,5-bisphosphate carboxylase (RuBisCO)-mediated carboxylation of RuBP and that the CO_2 fixation occurs via the Calvin cycle (C_3 cycle).

In several lichens, dark CO_2 fixation has also been noted.[104,107-109] In *P. aphthosa* dark CO_2 fixation rates represent 17 to 20% of that in light.[75] The cyanobiont and the mycobiont were both found to be capable of dark CO_2 fixation, and both contained phosphoenolpyruvate carboxylase (PEP Case). ^{14}C-kinetics data suggest dark CO_2 fixation to be via C_4 pathway.[75]

TRANSFER OF FIXED C

During light, a portion of the fixed C produced by the cyanobiont is made available to the mycobiont. In the dark, movement of fixed C from the cyanobiont to the mycobiont is extremely low or absent.[75,110] Among bipartite cyanolichens, the mycobiont depends entirely on the cyanobiont for its fixed-C requirements. Movement of fixed C from the cyanobiont to mycobiont has been shown to occur in bipartite cyanolichens containing *Nostoc (P. polydactyla*,[107,111-113] *Collema aruriculatum*,[112] *P. horizontalis*,[105] *Leptogium* sp.,[112] *Lobaria scrobiculata*,[112,114] *P. canina*,[105] *Sticta fuliginosa*,[112] *Sticta* sp.[112]), *Calothrix (Lichina pygmea)*,[112] *and Scytonema (Coccocarpia* sp.).[112] In all these cases, the fixed C moves from the cyanobiont to the mycobiont in the form of glucose.

In tripartite lichens the movement of fixed C from the cyanobiont to mycobiont has also been noted to be in the form of glucose; the species investigated include *Lobaria amplissima*,[112,114] *P. aphthosa*,[112] and *Solorina saccata*.[112] However, the overall contribution from the cyanobiont, with respect to the whole thallus requirements of fixed C, is negligible since the cyanobiont forms a very small proportion of the thallus in tripartite lichens.[112] Englund[115] has shown that *Nostoc* in *P. aphthosa* supplies only 2 to 3% of its fixed C to the mycobiont. Furthermore, fixed C has been found to accumulate in the cyanobiont of *P. aphthosa* cephalodia in the form of polyglucoside granules,[75] lipids,[116] and water-soluble glucose polymers.[117] High frequency of heterocysts (which are nonphotosynthetic) and reduced number of vegetative cells (photosynthetic), higher requirement of fixed C for energy provision to nitrogen-fixation process, and overall smaller proportion of cyanobiont in such lichens, together, may explain the low amount of fixed C transfer from cyanobiont to mycobiont in such lichens. In addition, it is desirable that the cyanobiont fixes more nitrogen (rather than carbon) in tripartite symbioses, since carbon is available from the phycobiont, while the small proportion of the cyanobiont has to provide fixed N to the whole thallus. A possibility has also been expressed that, the mycobiont in tripartite lichens, has a selective effect on the carbon metabolism of the phycobiont without affecting the carbon metabolism of the cyanobiont.[2]

The quantity of glucose transferred and the precise rates of such transfer are not known. Most studies involving ^{14}C tracers lack information regarding specific activities of $^{14}CO_2$ which is fixed and of fixed C which moves. Also, the proportion of transferred compound which is respired and the respired CO_2 which is refixed by the cyanobiont is also unknown.[118] In view of this, the rates described in literature as fast, intermediate, slow, and very slow have no realistic meaning.

The glucose, after its movement from the cyanobiont to mycobiont, is converted into mannitol in the mycobiont.[119,120] This serves as C reserve for the mycobiont and is not available to the cyanobiont.[2] Free-living fungi are unable to metabolize mannitol, which seems to be a specific metabolite of lichenized fungi.[2] Mannitol may also act as a physiological buffer during osmotic stress induced by drying-wetting cycles in nature.[121,122] In *P. aphthosa,* a tripartite lichen, little mannitol is synthesized in cephalodial hyphae.[117] This may be a reflection of the fact that little fixed C comes from the cyanobiont which occurs in cephalodia. In contrast, most mannitol synthesis occurs in fungal hyphae in the main thallus which are in direct contact with the phycobiont.

The precise mechanism of how the cyanobiont is modified to cause release of glucose, produced during photosynthesis, is not known. However, it seems that the mycobiont affects the C metabolism of the cyanobiont, at least in the bipartite lichens.[2] Indeed, when the cyanobiont is removed from the thallus, liberation of fixed C declines steadily and rapidly, with a concomitant increase in more and more fixed C remaining in the cyanobiont.[119] In the case of nitrogen transfer (see later), ammonia, derived from nitrogen fixation in the cyanobiont, is transferred to the mycobiont because of very low glutamine synthetase (GS) activity and synthesis in the cyanobiont. Whether there is an analogous repression of C-metabolism enzyme(s) in the cyanobiont, resulting in glucose release, is as yet unexplored. Hill[123] suggested that in *P. polydactyla* the cyanobiont cell wall synthesis is modified, causing accumulation of a glucan pool outside the plasmalemma. This is then hydrolyzed by an extracellular enzyme from the mycobiont. However, this mechanism cannot apply to phycobionts since polyols, the mobile carbohydrates of phycobionts, are not components of their cell walls.[4] Furthermore, Peveling and Hill[124] have shown that the glucan pool is located inside (rather than outside) the cyanobiont cells. Peveling[63] suggested that the hydrolysis of these glucans may result from action of lomasomal or lysosomal enzymes occurring adjacent to these structures in the cyanobiont. Smith[119] has suggested that mycobiont-induced reduction or reversal of the membrane potential of the photobiont may be the cause of carbohydrate efflux from photobionts. However, this hypothesis has been ruled out by Stewart et al.,[79] who studied aspects of membrane potential in *P. aphthosa* phycobiont. A more recent suggestion is that carbohydrate efflux from the photobiont is a carrier-mediated process.[4,120,125-129] Although the evidence for this is not conclusive, several lines of indirect evidences suggest this to be a distinct possibility.[120]

METHODS EMPLOYED TO STUDY PHOTOSYNTHATE TRANSPORT IN LICHENS

To demonstrate that fixed C moves from cyanobiont to mycobiont, the thallus, after exposure to $^{14}CO_2$, can be separated into cyanobiont-free and cyanobiont-containing regions of the lichen. A progressive accumulation of photosynthetically fixed ^{14}C in the photobiont-free region indicates photosynthate movement from photobiont to mycobiont.[130]

A method used for identification of the mobile carbohydrate involves physical separation of the cyanobiont by thallus homogenization and differential or gradient centrifugation.[107,113,131] In the freshly isolated cyanobiont, carbohydrate efflux slows down quickly and eventually stops, but the mobile carbohydrate is detectable initially. The method has several constraints, including difficulty in isolation of the cyanobiont, filament disruption, and inability to quantify the actual amount/rate of carbohydrate transfer.[120]

A method termed isotope trapping, or inhibition technique, has been extensively used to identify the mobile carbohydrate and the rate of such transfer.[111,112,120,132] Although the rates of transfer may not be precise,[120] for identification of mobile carbohydrate this technique has been very useful. Experiments consist of incubating a lichen in a solution of a range of different types of carbohydrates, adding $NaH^{14}CO_3$, and determining in which medium large amounts of fixed ^{14}C are released from the lichen.[132]

Other techniques used to study carbohydrate movement from cyanobiont to mycobiont have employed digitonin (which selectively disrupts fungal membranes releasing the mobile photosynthate into the medium),[129] microautoradiography (to localize distribution of fixed ^{14}C),[124,127] and various transport inhibitors (sorbose, 2-deoxy-glucose, uranyl ions, etc.).[120,127,129]

The fate of mobile carbohydrate, once it reaches the mycobiont, can be studied by feeding labeled mobile carbohydrate to the portions of lichen thalli containing only the mycobiont and subsequent analysis of the conversion product.

Various methods mentioned above have been reviewed and critically evaluated by Smith.[120] Richardson[133] has recently reviewed various aspects of carbohydrate transfer between lichen symbionts. For a more up-to-date and detailed coverage, see the publication by Galun.[5]

NITROGEN METABOLISM

NITROGEN CONTENT

Total nitrogen content (as percent of dry weight) has been analyzed in several lichens.[78,115,131,134-139] The data on cyanolichens are compiled in Table 1. In general, cyanolichens have higher N content than lichens with green algae. Hitch and Stewart[136] have reported an average N content of 2.2% in cyanolichens and 0.85% in lichens with green algae. Green et al.[135] have analyzed 22 species of *Pseudocephellaria,* 7 species of *Sticta,* and 15 species of lichens with green algae. They found an average N content of 3.4% in cyanolichens and 0.5% in others. In the tripartite lichens *P. aphthosa* and *Placopsis gelida,* the N content has been reported to be higher in cephalodia than the rest of the thallus.[78,115,136] This may be a reflection of the fact that the cyanobiont, which is the source of fixed N, is located in cephalodia. In *Peltigera aphthosa* and *P. canina,* relative distribution of the total thallus protein among various partners has also been studied.[78,140] In *P. aphthosa, Nostoc, Coccomyxa,* and the mycobiont constitute 6%, 14.4%, and 79.6% of the total thallus protein, respectively.[78] In *P. canina, Nostoc* and mycobiont constitute 36% and 64% of the total thallus protein, respectively.[140] *Nostoc* constitutes nearly 80% of the soluble protein in cephalodia of *P. aphthosa.*[140] Smith[139] has shown that the total nitrogen pool in *P. polydactyla* consists of 75% insoluble N and 25% soluble N. Ammonia, amino-N, and amide-N constitute nearly 50% of the soluble pool.

Several cyanolichens have been analyzed for their soluble amino acid composition. Some of the earliest studies were done on *Lobaria isidiosa, Lobaria subisidiosa,* and *P. canina.*[141-145] Glutamic acid and glutamine were found to be the most abundant soluble amino acids present. Glutamic acid has also been found to be the most abundant amino acid in the soluble-N pool of *Sticta sylvatica, Lobaria laetevirens,* and *L. pulmonaria.*[134] Rai et al.[146,147] have analyzed the ammonia and amino acid pools of *P. aphthosa* cephalodia, and *P. canina* thallus (Table 2). In *P. aphthosa* cephalodia, glutamate was most abundant followed by

TABLE 1
Total Nitrogen Content of Cyanolichens

Lichens	N content (% of dry wt)	Ref.
Coccocarpia cronia	4.80	135
Collema auriculatum	3.60	104
C. crustatum	4.39	104
C. fluviatile	4.37	104
C. furfuraceum	4.09	104
C. subconveniens	4.00	135
C. subfervum	3.85	104
Ephebe lanata	5.80	104
Leptogium burgessii	5.51	104
L. lichenoides	3.87	104
L. sinuatum	4.31	104
L. terestrinsculum	4.50	104
Lichina confinis	3.73	136
L. pygmea	6.49	104
Lobaria laetevirens	2.20	134
L. pulmonaria	2.70	134
L. scrobiculata	2.77	104
Massalongia carnosa	4.27	104
Nephroma australe	1.70	135
N. laevigatum	4.22	104
Pannaria microphylla	5.14	104
P. pezizoides	6.37	104
P. rubiginosa	2.25	104
Parmeliella atlantica	3.90	104
P. plumbea	4.76	104
Peltigera aphthosa (var. *leucophlebia*)	3.00	131
P. aphthosa Willd.		
Whole thallus	2.22—3.30	78,115,136
Excised cephalodia	2.69—6.44	78,115,136
Thallus without cephalodia	1.90—2.89	78,136
P. canina	3.30	137
P. dolichoriza	3.70	135
P. polydactyla	1.83—4.50	104,135,136,139
P. praetextata	4.70	138
P. rufescens	2.22	136
Peltigera sp.	4.70	135
Placopsis gelida		
Whole thallus	0.93	136
Excised cephalodia	1.74—2.59	136
Thallus without cephalodia	0.86—0.97	136
Placynthium nigrum	2.73	104
P. pannariellum	4.61	104
Polychidium muscicola	3.19	104
Pseudocephellaria aurata	2.30	135
P. billardierri	3.40	135
P. cf. carpoloma	2.20	135
P. colensoi	1.50	135
P. coriacea	3.40	135
P. coronata	1.60	135
P. crocata	3.30	135
P. degelii	2.80	135
P. delisea	2.60	135
P. dissimilis	5.20	135
P. faveolata	2.10	135
P. flavicans	2.60	135

TABLE 1 (continued)
Total Nitrogen Content of Cyanolichens

Lichens	N content (% of dry wt)	Ref.
P. granulata	2.20	135
P. hamata	4.20	135
P. hirsutula	3.10	135
P. homeophylla	2.60	135
P. hookeri	3.70	135
P. intricata	4.10	135
P. amphisticta	3.50	135
P. multifida	4.10	135
P. polychista	4.30	135
P. rubella	2.60	135
P. thouarsii	3.90	104
Sticta caperata	4.90	135
S. filix	4.20	135
S. fuliginosa	4.19—4.60	104,135
S. latifrons	3.20	135
S. limbata	3.93—5.20	104,135
S. variabilis	4.10	135
S. sylvatica	4.00	134
S. weigelii	4.70	135

TABLE 2
Pools of Ammonia and Major Amino Acids in the Thallus and Cyanobiont of *Peltigera canina* and in the Cephalodia of *P. aphthosa*[146,147]

Ammonia/amino acids	*P. canina* (μmol/g thallus dry wt) Whole thallus	*P. canina* (μmol/g thallus dry wt) Cyanobiont[a]	*P. aphthosa* (μmol/g cephalodial dry wt)
Alanine	145.20	29.10	29.00
Ammonia	33.80	10.80	18.00
Asparagine	3.80	Trace	Trace
Aspartate	58.70	6.10	11.00
Glutamate	76.00	28.80	50.00
Glutamine	93.00	34.00	10.00
Glycine	14.40	4.00	Trace
Serine	14.60	Trace	Trace
Threonine	11.00	Trace	Trace

Note: Values below 1 μmol are noted as Trace.

[a] Values obtained from digitonin-treated thalli.

alanine, ammonia, aspartic acid, and glutamine. In the case of *P. canina,* alanine was most abundant followed by glutamine, glutamate, aspartic acid, and ammonia. In the cyanobiont of *P. canina,* major pools were glutamine, alanine, glutamate, aspartic acid, and ammonia. The cyanobiont possessed a third of the total ammonia, glutamate, and glutamine pools of the thallus. This is as expected because the cyanobiont constitutes nearly a third of the total thallus on the protein basis. However, pools of aspartic acid and alanine are proportionately much lower in the cyanobiont. This indicates that much of the aspartic acid and alanine pools in *P. canina* thallus are located in the mycobiont.

Various amines have been reported to be common in several cyanolichens. In *L. lae-*

tevirens, methylamine, dimethylamine, and trimethylamine are abundant.[148,149] A scheme summarizing the interrelationships among glycine, methylamines, sarcosine, and choline has been proposed in this lichen.[149] Free di- and polyamines are reported to be involved in a number of physiological processes and a close relationship among polyamine, nucleic acids, and membranes has been suggested.[150] Polyamines protect nucleic acids, membranes, and ribosomes against thermal and enzymic destruction by binding and stabilizing them under extreme environmental conditions.[151,152] High polyamine content could also help lichens to overcome drought stress.[153,154]

In *P. praetextata,* sarcosine has been detected with a concentration of 34 nmol/mg dry thallus weight.[155] It has been suggested that sarcosine may play a role in regulation of the primary ammonia-assimilating enzyme GS.[155] Another soluble nitrogenous compound, sticticin, has been detected in *L. laetevirens.*[156] At a thallus water content of 10 to 12%, sticticin concentration may exceed 1 *M* in the thallus and may play an osmoregulatory role.[156]

A group of nitrogenous compounds commonly found in cyanobacteria are the phycobiliproteins.[157] Stewart et al.[79] found that cultured cyanobiont of *P. aphthosa* contained both phycoerythrin and phycocyanin, while the cyanobiont in cephalodia had only phycoerythrin. Czeczuga[80] later reported the presence of phycocyanin and phycoerythrin in *P. aphthosa, P. canina, P. degenii, P. polydactyla,* and *P. praetextata. P. horizontalis* and *P. rufescens* contained allophycocyanin in addition to phycocyanin and phycoerythrin.[80]

UTILIZATION OF EXOGENOUS COMBINED NITROGEN

Uptake and utilization of exogenously supplied ammonia, nitrate, and amino acids have been studied in several cyanolichens.[139,158-168] Solomon[158] reported that lichens with green algae take up much more nitrate, on a per unit thallus weight basis, than those with cyanobionts, although both types had similar rates of ammonia absorption. In *P. polydactyla,* nitrate absorption was found to be 20% of the ammonia absorption from a solution of similar concentration (5 m*M*).[139] Furthermore, virtually all the nitrate absorbed remained as nitrate in the thallus.[139]

P. polydactyla has been shown to absorb ammonia at a rate of 400 ng ammonia-N per hour per milligram thallus dry weight.[139] The rate of absorption decreases with increase in time and ceases after 12 h. The rate of absorption is enhanced by the addition of glucose.[139] Ammonia absorption leads to an increase in ammonia and amino-N content of the thallus, but it does not affect the amide-N content.[139] This has led to the suggestion that amides do not have an important quantitative role in the N metabolism of *P. polydactyla.*[139] Protein synthesis was found to be slow, in small amounts, and increased slightly with the addition of glucose.[139] In *P. aphthosa,* ammonia uptake from a 5-m*M* ammonium chloride solution was found to be 59 and 53 nmol/mg fresh weight per 24 h for the whole thallus and isolated cephalodia (3.6 and 3.12 mg ammonium-N per gram dry weight per 24 h), respectively.[159] $^{15}NH_4Cl$ uptake in *P. aphthosa* and *P. canina* showed rates of 2 and 1.5 mg ammonium-N per gram thallus dry weight per hour, respectively.[160] Uptake of ammonium by the cyanobionts were found to be much slower than those by the cultured isolates. Cyanobionts in the thallus took up ammonium at rates which were only 4 to 5% of those in cultured isolates. This extremely slow rate of ammonium uptake by the cyanobionts is apparently due to very low levels of glutamine synthetase, the primary ammonia-assimilating enzyme.[161-163] It has also been suggested that cyanobionts in lichens may lack an ammonium transport system.[164] Although such studies are lacking on lichen cyanobionts, studies on *Azolla* cyanobiont suggest that the ammonium transport system is present.[165] Recently, however, an ammonium transport system has been found to be lacking in the cyanobiont of *Cycas.*[166]

Uptake and utilization of asparagine, glutamine, aspartic acid, and glutamate have been studied in *P. polydactyla.*[167,168] Asparagine uptake was found to be faster than glutamine, aspartic acid, and glutamate. Absorption of all these amino acids was accompanied by

TABLE 3
Activities of Some Enzymes Involved in Nitrogen Metabolism of Some Cyanolichens[a]

Lichen	Alanine dehydrogenase (EC 1.4.1.1)	Glutamate dehydrogenase (EC 1.4.1.4)	Fd-dependent glutamate synthase (EC 1.4.7.1)	Glutamine synthetase (EC 6.3.1.2)
Peltigera aphthosa				
Whole thallus	9.70	90.00	—	25.70
Excised cephalodia	8.50	398.00	0.85	3.50
Thallus without cephalodia	10.00	35.00	—	17.50
Cyanobiont	—	0.00	0.72	4.00
Phycobiont	—	—	20.94	40.00
Cultured cyanobiont	11.00	2.00	31.29	70.00
Cultured phycobiont	3.00	22.00	23.10	37.00
Mycobiont	15.00	24.00	0.00	0.00
P. canina				
Whole thallus	3.80	195.80	0.28	0.80
Cyanobiont	5.50	2.40	0.84	4.10
Cultured cyanobiont	22.90	0.00	33.50	72.40
Mycobiont	—	—	0.00	0.00
Lobaria laetevirens	—	370.00	—	—

Note: — = not determined.

[a] Compiled from References 3, 78, 146, 147, 159, 160, 170, 171, and 173.

significant increases in the amounts of ammonia and amino-N of the thallus. Within the thallus, the cyanobiont region absorbs more asparagine and ammonia than the medulla, suggesting higher metabolic activity in the cyanobiont region.[168] The utilization of absorbed asparagine is very slow and involves deamination of asparagine to ammonia.[167] Asparagine uptake is adversely affected by glucose.[167] The high absorptive ability and slow utilization of accumulated nutrients may be a useful metabolic adaptation in lichens, enabling accumulation of nutrients during periods of abundance and their use during periods of scarcity.[167]

ENZYMES OF NITROGEN METABOLISM

Many lichens have urease activity and available data suggest that urease is a constitutive enzyme in cyanolichens, while in lichens with green algae it is urea-inducible.[160,169]

Carbamoyl phosphate synthase (CPS) activity, both ammonia- and glutamine-dependent, has been studied in *P. aphthosa.*[75] Glutamine was found to be a poorer substrate for this enzyme than ammonia. Cephalodial extracts showed higher CPS activity than the main thallus. In cephalodia, ammonia-dependent CPS activity was found to be 1.7 and glutamine-dependent activity 0.25, nmol product formed per minute per milligram protein. In contrast, the main thallus showed ammonia-dependent CPS activity of 0.1, and glutamine-dependent activity of 0.05, nmol product formed per minute per milligram protein.

Alanine dehydrogenase (ADH) activity has been studied in *P. aphthosa* and *P. canina* (Table 3).[146,147,159,170] In *P. canina,* the cyanobiont ADH activity is much lower than that in the cultured isolate. In the case of *P. aphthosa,* the ADH activity in whole thallus, cephalodia, and thallus-without-cephalodia is comparable, although the mycobiont contains significantly higher activity.

Glutamate dehydrogenase (GDH) activity has been studied in the lichens *P. canina, P. aphthosa,* and *L. laetevirens* (Table 3).[146,147,159,171] In *L. laetevirens,* two NADP-dependent GDH have been detected, of which the major one is located in the mycobiont. The enzyme has been purified and found to be more active in glutamate biosynthesis than in glutamate oxidation. Its activity is dependent upon ammonia concentration, ionic strength, and pH.[171]

In *P. canina* and *P. aphthosa,* higher NADP-dependent GDH activity has been reported in the mycobiont.[146,147,159,170] The cyanobiont has little or no GDH activity. In *P. aphthosa,* the cephalodial mycobiont has higher GDH activity than the medullary fungal hyphae.[146,159] This is important since it is the cephalodial hyphae which are in close contact with the nitrogen-fixing cyanobiont cells. Indeed, within the cephalodia, the GDH activity is much more intense in regions of cyanobiont-mycobiont contact.[78,170] In *P. canina* and *P. praetextata* as well, the GDH activity localization shows a much higher level in the fungal hyphae which are in close contact with the cyanobiont.[100,170] In *P. canina,* GDH activity distribution in different parts of the thallus shows lower activity in the apex region.[172]

Glutamate synthase (GOGAT) activity has been examined in *P. canina* and *P. aphthosa* (Table 3).[173] Fd-dependent GOGAT activity was detected in the cyanobiont of *P. canina* and *P. aphthosa,* but the level was only about 3% of that present in the respective cultured isolates. In both lichens, no GOGAT activity (either Fd-dependent or NADH/NADPH-dependent) was found in the mycobiont. In contrast, the phycobiont *(Coccomyxa)* had Fd-dependent GOGAT activity similar to that in the cultured isolate. How GOGAT activity is affected in the cyanobiont is not clear at present but, like the GS activity (see later), only the GOGAT of the cyanobiont seems to be affected.

GS activity and its localization in *P. canina* and *P. aphthosa* has been studied in detail.[146,147,159,170,172,174,175] In both cases, GS activity of the cyanobiont has been reported to be reduced by over 90% as compared to their cultured counterparts (Table 3). The reduction in activity was earlier thought to be due to inactivation of the enzyme,[140] but later it was established that this is due to low levels of GS protein.[78,174,175] The precise mechanism of the modification of GS synthesis is as yet unknown in lichens. However, in *Azolla,* the transcription of GS structural gene has been shown to be affected.[176] The effector which causes the change in the GS levels of the cyanobiont is also not known. Sarcosine has recently been proposed as one of the compounds which may be involved in regulation of GS levels in *P. praetextata.*[155] The low GS activity present in the cyanobionts is not distributed uniformly throughout the thallus. Indeed, much of the activity is in the growing apical region of the thallus while little or none is present in older parts.[172] Immunogold localization studies of Hallbom et al.[175] suggest that the residual GS protein present in the cyanobiont is evenly distributed in heterocysts and vegetative cells. This is in contrast to the distribution pattern in the free-living isolates, where heterocysts contain a GS concentration twofold higher than that in vegetative cells. This suggests that there is a greater reduction of GS in heterocysts of the cyanobionts in comparison to their vegetative cells. GS activity is undetectable in any of the mycobionts. However, the phycobiont *Coccomyxa* shows GS activity similar to that in the cultured isolate. This shows that the reduction in GS levels is selective since only the cyanobiont GS level is affected.

It is interesting to note here that, as in lichens, there is a selective reduction in GS protein level of heterocysts in cyanobionts of *Anthoceros* and *Azolla* symbioses.[175a] A similar situation is found in those few heterocysts that remain in ammonium-grown cultures of *Anabaena cylindrica.*[175a]

The earliest reports of aminotransferase activities in lichens are those of Bernard[177] and Bernard and Goas,[178] who studied glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and glutamate decarboxylase activities in some members of the Stictaceae. GOT was found to be the major transaminating enzyme and its activities were higher than GPT. GOT, GPT, and aspartate pyruvate transaminase (AsPT) activities in *P. canina* have been reported (Table 4).[147,170] Activities of all the three aminotransferases in the cyanobiont were significantly lower than that in the cultured isolate. Furthermore, on digitonin treatment, the specific activity of these aminotransferases left in the thallus is reduced significantly. Since digitonin selectively disrupts fungal membranes, the residual activity is thought to be that of the cyanobiont. Thus, it has been suggested that the specific

TABLE 4
Activities of Serine Transhydroxymethylase and Aminotransferases in *Peltigera aphthosa* and *P. canina*[a]

Lichen	Serine transhydroxymethylase (EC 2.1.2.1)	Glutamate glyoxylate transaminase (EC 2.6.1.4)	Glutamate oxaloacetate transaminase (EC 2.6.1.1)	Glutamate pyruvate transaminase (EC 2.6.1.2)	Aspartate pyruvate transaminase (EC 2.6.1.12)
P. aphthosa					
Cephalodia	1.50	44.50	20.20	40.10	15.82
Cyanobiont	—	—	12.50	0.00	2.60
Thallus without cephalodia	2.90	56.00	47.00	—	—
Phycobiont	3.20	14.50	—	—	—
Mycobiont (medulla)	2.20	—	37.00	—	—
Whole thallus	—	—	58.40	—	—
Cultured cyanobiont	—	—	25.00	4.80	—
Cultured phycobiont	—	—	520.00	—	—
P. canina					
Whole thallus	—	—	41.00	18.12	126.00
Cyanobiont	—	—	13.80—16.80	0.86	58.60
Cultured cyanobiont	—	—	40.40	—	—

Note: — = not determined.

[a] Data compiled from 3, 146, 147, 159, 160, and 170.

activity of these enzymes is significantly higher in the mycobiont.[147,170] In *P. aphthosa,* GOT, GPT, AsPT, glutamate-glyoxylate transaminase, and serine transhydroxymethylase activities have been reported.[3,146,159,160,170] GOT is present both in the cyanobiont and in the mycobiont, the latter having higher specific activity.[146,170] The cultured phycobiont *Coccomyxa* shows very high GOT activity. High GPT activity is present in cephalodia and it is located in the mycobiont.[146] AsPT activity also is located primarily in the mycobiont.[146] Glutamate-glyoxylate transaminase activity is similar in cephalodia and in the main thallus; the phycobiont has very low activity.[160] Serine transhydroxymethylase activity, on the other hand, is highest in the phycobiont and very low in the cyanobiont.[160]

HETEROCYST FREQUENCY

Under aerobic conditions, heterocysts are the sites of nitrogen fixation in heterocystous cyanobacteria.[179,180] In free-living cyanobacteria, the heterocyst frequency is normally about 5%. In lichens, however, the heterocyst frequency of the cyanobiont is altered. Although heterocyst frequency in bipartite lichens is normally similar to that in free-living cyanobacteria, in tripartite lichens the cyanobiont has a heterocyst frequency which is much higher than that in free-living cyanobacteria (Table 5). Heterocyst frequency of the cyanobiont may vary within the lichen thallus. In *P. aphthosa,* heterocyst frequency of the cyanobiont in the apical region of the thallus is lower than that in the central or basal regions of the thallus.[115]

The precise explanation for increase in heterocyst frequency of the cyanobionts in tripartite lichens is lacking. However, some possible explanations include:

1. Cyanobionts in tripartite lichens develop a high percentage of heterocysts, which are nonphotosynthetic, because the fixed-C requirements of the mycobiont are met by the phycobiont and the fixed-C requirements of the cyanobiont are lower (compared to that in the free-living form) due to slow growth.[170] How increased heterocyst differentiation is triggered remains to be worked out.

TABLE 5
Heterocyst Frequency in Cyanobionts of Cyanolichens[a]

Lichens	Heterocyst frequency (%)
Bipartite	
Collema auriculatum	2.70
C. crustatum	2.10
C. furfuraceum	4.00
C. subfervum	2.60
Leptogium burgessii	4.50
L. cyanescens	2.70
L. lichenoides	2.60
L. sinuatum	2.40
L. tremelloides	3.50
Lichina confinis	2.40
L. pygmea	4.60
Lobaria scrobiculata	3.90
Massalongia carnosa	5.50
Nephroma laevigatum	4.10
N. perile	4.90
Pannaria microphylla	4.50
P. pezizoides	3.90
Parmeliella atlantica	7.30
P. plumbea	5.90
Peltigera canina	4.70
P. evansiana	4.70
P. polydactyla	5.80
P. praetextata	4.40
P. venosa	7.80
Placynthium nigrum	2.00
Polychidium muscicola	2.60
Pseudocephellaria thouarsii	6.70
Sticta fuliginosa	6.00
S. limbata	4.90
S. sylvatica	3.50
Tripartite	
Dendriscocaulon umhausense	1.90—15.20
Lobaria amplissima	21.60
L. laetevirens	30.40
L. pulmonaria	35.60
Nephroma arcticum	14.10
Peltigera aphthosa	21.20
Placopsis gelida	15.00
Psoroma hypnorum	10.10
Solorina crocea	17.80
S. saccata	14.70
S. spongiosa	5.80—54.70
Stereocaulon vesuvianum	20.50

[a] Compiled from References 104 and 181.

2. High heterocyst frequency in cyanobiont of tripartite lichens may be caused by stimulation of heterocyst formation due to photosynthate movement from phycobiont to the cyanobiont.[26] This possibility remains to be explored experimentally.
3. In symbiosis, an effector may be produced by the mycobiont which causes increased heterocyst differentiation in the cyanobiont. Here too the identity of the effector and the discrimination between cyanobionts of bipartite and tripartite lichens remain to be explored.

In two bipartite lichens, *Heppia echinulata* (cyanobiont — *Scytonema*) and *Placynthium nigrum* (cyanobiont — *Calothrix*/*Dichothrix*), the cyanobionts have been reported to become unicellular, losing their filamentous character and heterocysts in symbiosis.[8,15]

NITROGEN FIXATION

All lichens with heterocystous cyanobionts fix nitrogen. A list of lichens so far tested for nitrogen fixation, using acetylene reduction technique or $^{15}N_2$ incorporation, is given in Table 6.

Nitrogen fixation rates in lichens vary considerably. Factors which are known to affect nitrogen fixation rates are pH, temperature, light intensity, thallus moisture content, season of lichen collection, environmental conditions prevailing before time of collection, and the period of pretreatment and/or storage.[104,196] Variation in nitrogen-fixation rates are also likely due to variation of heterocyst frequency in different replicate thalli, or even in different parts of the same thallus. For these reasons, it is not possible to have a meaningful comparison of nitrogen-fixation rates reported in various lichens. Optimum rates of nitrogen fixation in the *P. canina* cyanobiont have been reported to be about 8 nmol acetylene reduced per microgram chlorophyll *a* per hour. This is comparable to the activity in the cultured isolate (9.4 nmol acetylene reduced per microgram chlorophyll *a* per hour).[160,196] In *P. aphthosa,* the optimum rate has been found to be 21 nmol acetylene reduced per microgram chlorophyll *a* per hour. This is considerably higher than that in the cultured isolate (9 nmol/μg chlorophyll *a* per hour).[170] Nitrogenase activity has been shown to vary in different parts of the *P. canina*[136,172] and *P. aphthosa*[115] thalli. In *P. canina,* nitrogen fixation rates are highest toward the tip of the thalli and lowest towards the base.[136,172] In *P. aphthosa,* the apical region of the thallus has low nitrogenase activity; highest nitrogenase activity occurs in the central portion of the thallus.[115] This has been correlated with heterocyst frequency which is lower in the apical region and higher in the central parts of the thallus.

On removal from the thallus, the rates of nitrogen fixation by cephalodia have been reported to be adversely affected in *Stereocaulon paschale.*[27] However, in *P. aphthosa,* nitrogenase activity of cephalodia was not affected on removal from the thallus.[146]

There has been an earlier suggestion that in *P. canina,* nitrogen fixation may occur even in vegetative cells of the cyanobiont.[137] However, recent studies on nitrogenase localization, using immunogold labeling, suggest that nitrogenase is located in heterocysts only.[180] Most studies measuring nitrogen-fixation rates have used acetylene reduction technique because of the ease and speed of sample analysis. However, in order to relate rates of acetylene reduction to actual nitrogen fixation, it is necessary to determine the ratio between acetylene reduction and $^{15}N_2$ fixation. This is mainly because the ratio varies widely (from the theoretical value of three) due to various reasons.[197-202] For *P. aphthosa,*[78] *P. canina,*[147] *L. pulmonaria,*[203] and *L. oregana,*[203] a ratio of four to five has been reported. However, ratios of up to 20 have been reported by Millbank.[192] Some of these variations between studies of Millbank[192] and those of others[78,147,203] may be due to differences in their experimental design,[160] but a significant variation from the theoretical ratio of three has been noted in all studies. This emphasizes the need to run ^{15}N experiments to determine the actual ratios between acetylene reduction and nitrogen fixation before the acetylene reduction data are extrapolated in terms of nitrogen fixation. Another problem associated with acetylene reduction assay has been noted in *P. polydactyla.* MacFarlane et al.[204] have found that on prolonged incubation of thalli with acetylene, the rate of acetylene reduction increases with increase in the incubation time. After 100 h of exposure to acetylene, levels of acetylene reduction were sixfold higher than those in the thalli exposed to air between incubations. Such a phenomenon has been noted in free-living cyanobacteria also, and Stewart[205] has suggested that this stimulation is probably due to the nitrogen depletion (in the presence of acetylene there is no N_2 reduction) which results in an increased nitrogenase synthesis. Thus,

TABLE 6
Established Nitrogen-Fixing Cyanolichens[a]

Lichens	Ref.	Lichens	Ref.
Coccocarpia cronia	135	*P. pruinosa*	18
Collema auriculatum	26	*P. rufescens*	186
C. coccophorus	182	*P. scrabosa*	193
C. crispum	136	*Peltigera* sp.	135
C. flaviatile	26	*Placopsis gelida*	136
C. furfuraceum	26	*Placynthium nigrum*	26
C. granosum	183	*P. pannariellum*	26
C. pulposum	184	*Polychidium muscicola*	26
C. subconveniens	135	*Pseudocephellaria amphisticta*	135
C. subfervum	26	*P. aurata*	135
C. tenax	185	*P. billardierri*	135
C. tuniforme	186	*P. cf. carpoloma*	135
Ephebe lanata	26	*P. colensoi*	135
Heppia echinulata	2	*P. coriacea*	135
Lecidea sp.	185	*P. coronata*	135
Leptogium burgessii	26	*P. crocata*	135
L. cyanescens	187	*P. degelii*	135
L. lichenoides	183	*P. delisea*	135
L. sinuatum	26	*P. dissimilis*	135
L. terestiusculum	26	*P. faveolata*	135
Lichina confinis	188	*P. flavicans*	135
L. pygmea	188	*P. granulata*	135
Lobaria laetevirens	26	*P. hamata*	135
L. oregana	189	*P. hirsutula*	135
L. pulmonaria	190	*P. homeophylla*	135
L. scrobiculata	26	*P. hookeri*	135
Massalongia carnosa	26	*P. intricata*	135
Nephroma arcticum	194	*P. multifida*	135
N. australe	135	*P. polyschista*	135
N. laevigatum	26	*P. rubella*	135
Pannaria pezizoides	26	*P. thouarsii*	26
P. rubiginosa	26	*Solorina crocea*	194
Parmeliella atlantica	26	*Stereocaulon paschale*	195
P. plumbea	26	*Sticta caperata*	135
Peltigera aphthosa	131	*S. filix*	135
P. canina	190	*S. fuliginosa*	26
P. dolichoriza	135	*S. latifrons*	135
P. evansiana	191	*S. limbata*	26
P. membranacea	192	*S. variabilis*	135
P. polydactyla	18	*S. weigelii*	187
P. praetextata	138		

[a] In the following genera heterocysts have been detected, but nitrogenase activity has not been tested by using acetylene reduction or $^{15}N_2$ incorporation: *Dendriscocaulon umhausense, Collema crustatum, Leptogium tremelloides, Lobaria amplissima, Nephroma perile, Pannaria microphylla, Peltigera venosa, Psoroma hypnorum, Solorina saccata, S. spongiosa, Sterocaulon vesuvianum, Sticta dufourii,* and *S. canariensis.*[26,104]

in experiments involving nitrogenase assays using acetylene reduction technique, short-term assays should be preferred and the enhancement effects of acetylene on acetylene reduction rates over a prolonged period should be kept in view in interpreting data.

REGULATION OF NITROGEN FIXATION

Various factors affect nitrogen fixation in lichens. Aspects of environmental control of nitrogen fixation in lichens have been reviewed in great detail by Kershaw.[104] Here the regulation of nitrogenase by Mo and combined nitrogen, and certain regulatory aspects of dark nitrogen fixation, are discussed.

Mo is a key nutritional requirement for diazotrophs and it is a constituent of nitrogenase protein. Investigations of Horstmann et al.[203] on *L. pulmonaria* and *Lobaria oregana* have shown that addition of 1 ppm Mo enhances their nitrogenase activity drastically (180% enhancement in *L. pulmonaria* and 50% enhancement in *L. oregana*). This suggests that optimum levels of Mo may not be available to these lichens in nature. Indeed, Horstmann et al.[203] detected only 0.01 ppm Mo levels in water falling through the Douglas fir trees from where the lichens were collected.

In *P. aphthosa* and *P. canina*, regulation of nitrogenase by combined nitrogen has been investigated in detail.[78,159,170,206] Nitrogenase activity in *Nostoc* CAN (the cultured cyanobiont of *P. canina*) is fully inhibited by 1 to 2 m*M* ammonium chloride, while the inhibition of nitrogenase activity of the cyanobiont in *P. canina* thallus is only about 45%.[170] Nitrogenase activity in *Nostoc* CAN is inhibited 50 to 60% by 1 to 2 m*M* potassium nitrate, while no inhibition is detected in the cyanobiont in *P. canina* thallus.[170] This partial effect, or lack of effect, of combined nitrogen on nitrogenase activity of the cyanobiont in *P. canina* thallus is probably due to very low rates of assimilation of these compounds by the cyanobiont (since GS activity is extremely low).[147,170] Similarly, ammonium chloride does not affect nitrogenase activity of the cyanobiont in excised cephalodia of *P. aphthosa,* whereas in *Nostoc* APH (the cultured cyanobiont of *P. aphthosa*) nitrogenase activity is totally inhibited by ammonium chloride.[159] Such an inhibition is also observed when intact cephalodia (i.e., *P. aphthosa* discs containing cephalodia) are used. This inhibition can be overcome by the addition of L-methionine-DL-sulfoximine (MSX), which inhibits phycobiont GS located in the main thallus. Rai et al.[159] have suggested that addition of exogenous ammonia to discs leads to production of high levels of glutamine via GS in the phycobiont. This glutamine accumulates throughout the thallus, including the cephalodia where it inhibits cyanobiont nitrogenase. Indeed, liberation of glutamine by phycobiont has been shown to occur in the presence of excess exogenous ammonia.[160] When GS of the phycobiont is inactivated by MSX, such an inhibition does not occur due to nonproduction of glutamine. In case of excised cephalodia, lack of nitrogenase inhibition by ammonia may be explained similarly. Since cyanobiont has extremely low GS activity and mycobiont has none, ammonia assimilation via GS is very little.

Recent studies have indicated that ammonia per se can also inhibit nitrogenase in cyanobacteria.[207,208] Studies on *P. aphthosa* also have indicated that ammonia, and a product of ammonia assimilation via GS, may exert independent inhibitory effect on nitrogenase in the cyanobiont.[206] However, ammonia per se is inhibitory in *P. aphthosa* only when allowed to accumulate in high concentrations, e.g., in absence of CO_2 in darkness.[206] Normally, inhibition of nitrogenase by ammonia does not occur because (1) ammonia does not accumulate because much of it is assimilated by mycobiont GDH and (2) due to low GS activity, the cyanobiont assimilates very little ammonia.

Thus, both the ammonia levels and the levels of the product of its assimilation via GS remain below the inhibitory level. Even in free-living cyanobacteria, there is evidence to suggest that ammonia causes little or no inhibition of nitrogenase when GS activity is low.[162,209,210] There has been a suggestion that lack of inhibition of nitrogenase by ammonia in symbiotic cyanobacteria may be due to inability of the ammonium to enter the cell if the ammonium transport system is missing.[164] However, this is highly unlikely.[160]

P. aphthosa has been shown to fix nitrogen in darkness as well as in light.[75] Excised cephalodia and digitonin-treated cephalodia are also capable of dark nitrogen fixation to a similar extent as the whole thallus.[206] This suggests that the mycobiont and the phycobiont are not essential as sources of energy or reductant for dark nitrogenase activity in the cyanobiont. It also rules out the possibility of operation of a malate-aspartate-type shuttle between the symbionts for provision of reductant to the cyanobiont, as reported in *Alnus*.[211] Dark nitrogenase activity in the cyanobiont is supported by the catabolism of glycogen reserves accumulated in light, which in darkness serves to supply reductant and ATP.[75] This is supported by the following facts:

1. Glucose content of cephalodia decreases as the dark incubation period increases and the gycogen granules, which are present in the cephalodial cyanobiont, disappear after dark incubation.[75]
2. *Coccomyxa,* the only other phycobiont in the thallus, seems unimportant as a source of fixed C for the cyanobiont because both the light and dark nitrogenase activities of cephalodia are unaffected when they are removed from the *Coccomyxa*-containing main thallus.[75,206]
3. Dark nitrogenase activity in digitonin-treated cephalodia is as high and as prolonged as in intact cephalodia. This indicates that the mycobiont is not essential as a source of fixed C for reductant or energy supply to the cyanobiont.[206] Indeed, the cyanobiont is photosynthetic and even provides some fixed C to the mycobiont.[112,117]

The accumulation of enough polyglucose in the cyanobiont to support prolonged dark nitrogen fixation seems surprising, at first, considering the fact that in symbiosis a larger proportion of cyanobiont cells become nonphotosynthetic heterocysts. However, there are several factors which may facilitate accumulation of large amounts of polyglucose in the cyanobiont, during light, which will be sufficient for supporting dark nitrogenase activity over a prolonged period.[75,110,146,206] These include:

1. A reduced level of demand for fixed C for vegetative cell metabolism since the cyanobiont grows very slowly.
2. In the cyanobiont, a low requirement of carbon skeletons for ammonia assimilation because most of the ammonia produced during nitrogen fixation is liberated by the cyanobiont and assimilated by the mycobiont.
3. Less of a drain, by mycobiont, on fixed C produced by the cyanobiont because much of the fixed C requirement of mycobiont is met by the phycobiont *(Coccomyxa).*
4. A further reduced transfer of fixed C from the cyanobiont to the mycobiont during darkness.

Rai et al.[75,206] have shown that, in addition to the availability of fixed C from glycogen degradation, dark CO_2 fixation is also essential for prolonged nitrogenase activity in darkness. Dark CO_2 fixation occurs via PEP Case and provides carbon skeletons for ammonia assimilation in the mycobiont by replenishing the tricarboxylic acid cycle. In the absence of dark CO_2 fixation, ammonia accumulates and inhibits nitrogenase. When such ammonia accumulation is prevented by treating cephalodia with digitonin, dark nitrogenase activity continues in the absence of dark CO_2 fixation.[75,206]

The ability of the cyanobiont in *P. aphthosa* to fix nitrogen in light as well as in darkness may be important in meeting fixed-N requirements of other symbionts during brief periods of growth, when favorable growth conditions prevail in nature.

LIBERATION OF FIXED NITROGEN BY THE CYANOBIONT

Henriksson[212] showed that the *Nostoc* isolate of *C. tenax* releases substantial amounts of fixed N. Scott[138] later confirmed that this actually happens in the symbiosis. Subsequently, transfer of fixed N from cyanobiont to other symbionts was shown to occur in *P. aphthosa*[131,146,159] and *P. canina.*[147,170] In *P. canina,*[147,170] 50 to 55% of the nitrogen fixed by the cyanobiont has been shown to be transferred to the mycobiont, while in *P. aphthosa* up to 95% of the cyanobiont-fixed nitrogen is transferred to the other symbionts.[146,159]

TABLE 7
$^{15}N_2$ Fixation by Digitonin-Treated *Peltigera canina* Thallus Discs and the Distribution Pattern of ^{15}N[a]

Material	% of total fixed $^{15}N_2$ in each sample
Discs	44.50
Extracellular amino acids (organic N)	1.80
Extracellular ammonia	53.70

[a] *P. canina* thalli were incubated in a tenfold dilution of BG-11$_0$ medium[10] containing 0.01% (w/v) digitonin for 18 h. Discs of 1 cm in diameter were then cut, washed, resuspended in fresh medium, and exposed to $^{15}N_2$ for 6 h, followed by determination of ^{15}N in discs and in ammonia and organic N liberated into the medium. For further details see Reference 147.

The forms in which fixed N is released from the cyanobiont was first investigated by Millbank[213] using *P. aphthosa, P. canina, P. polydactyla, L. amplissima,* and *P. gelida.* A mixture of nitrogenous compounds, largely peptides, were suggested to be the compounds released by the cyanobiont. However, these may not truly reflect the nature of fixed N released by the cyanobiont since the experiments were done using intact thalli or cephalodia and the fungal metabolism may have modified the original nature of the fixed N released by the cyanobiont.[147,160,170] Later investigations by Stewart and Rowell[170] showed that in *P. canina,* ammonia was a major compound liberated by the cyanobiont. Even there, considerable amounts of fixed N were liberated in the form of organic nitrogen because the mycobiont metabolism was not inhibited totally.[147,160] In more carefully designed experiments, Rai et al.,[147] using ^{15}N tracer, have shown that the *P. canina* cyanobiont releases fixed N exclusively in the form of ammonia. Of the total fixed N, about 44% remained in the cyanobiont and the rest (55%) was released. Nearly 97% of the released nitrogen was in the form of ammonia (Table 7).[147] Similar experiments on *P. aphthosa* indicate that nearly 95% of the total fixed N is liberated by the cyanobiont and that the liberated nitrogen is almost exclusively in the form of ammonia.[146,159] Millbank and Kershaw[131] and Kershaw and Millbank[214] showed that fixed N is transferred from *P. aphthosa* cyanobiont to mycobiont and phycobiont both and that the phycobiont gets only 3% of its expected share of the fixed N. This led to the suggestion that the cyanobiont was of minimal value as a source of fixed N to the phycobiont.[214] However, later studies by Rai et al.[146,159] showed that *Coccomyxa,* the phycobiont, receives more than 5.2% of the total nitrogen fixed by the cyanobiont (Figure 1). This represents over 60% of the expected share of phycobiont.[146,170]

REASONS FOR THE LIBERATION OF FIXED NITROGEN

In cyanobacteria, assimilation of newly fixed ammonia occurs via the GS-GOGAT pathway.[163] GS is located in both heterocysts and vegetative cells and GOGAT only in vegetative cells.[215,216] In lichens, the GS and GOGAT activity of the cyanobiont is reduced by over 95%, with less than 5% normal activity remaining.[159,170,174] Based on such observations, it has been suggested that the liberation of fixed N by the cyanobiont, which is almost exclusively in the form of ammonia, is due to the inability of the cyanobiont to assimilate all the newly fixed ammonia being generated by nitrogen fixation because of insufficient levels of GS.[147,159,173] The fact that fixed N is liberated in the form of ammonia supports this view, as do the ^{15}N tracer studies on *P. aphthosa*[146] and *P. canina.*[147] In *P. canina,* the flux of ^{15}N through the amide-N of glutamine is considerably less than the rate of ^{15}N displacement from ammonia, and the flux of ^{15}N through glutamate is far greater than ^{15}N displacement from amide-N of glutamine. It is therefore evident that not all the ammonia generated by nitrogen fixation is assimilated in the cyanobiont of *P. canina.* A similar conclusion has been reached in the case of *P. aphthosa* cephalodia in which routes of nitrogen metabolism were studied using $^{15}N_2$.[146]

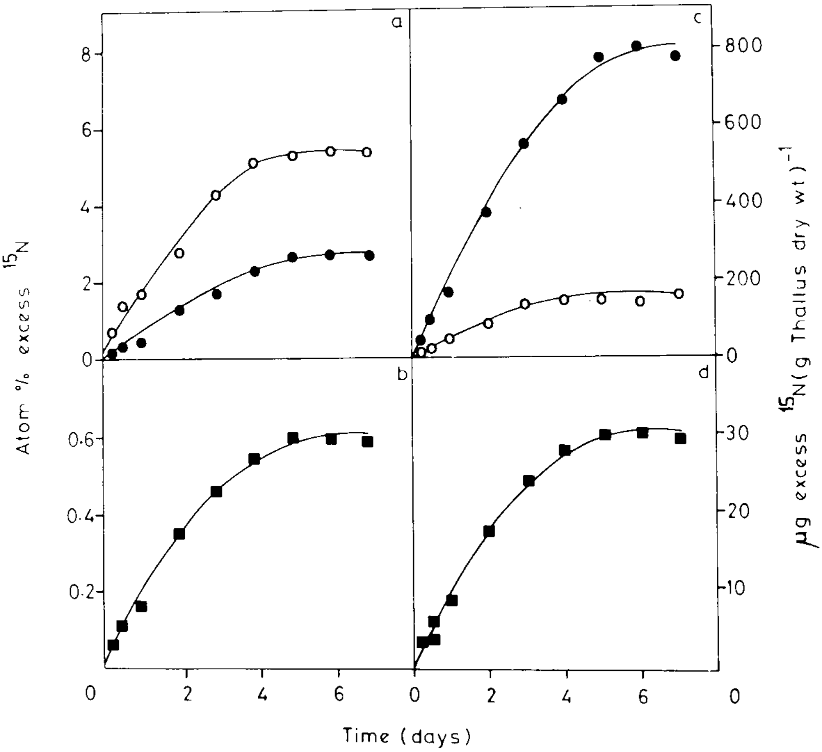

FIGURE 1. $^{15}N_2$ fixation and distribution pattern of ^{15}N in various components of *Peltigera aphthosa*. (a) and (b) ^{15}N labeling of each component (atom % excess ^{15}N. (c) and (d) Total ^{15}N label present in each component (µg ^{15}N per gram cephalodial dry weight). Symbols: ● = main thallus; ○ = cephalodia; ■ = *Coccomyxa*. Cephalodia-containing discs were incubated with $^{15}N_2$ and after various time intervals ^{15}N was analyzed in various components of the lichen thallus. For further details see Reference 78.

In both *P. aphthosa* and *P. canina*, the specific activities of GS and nitrogenase are comparable, and thus may be expected to assimilate all the ammonia produced during nitrogen fixation.[147,159,173] However, as previously discussed, we know that this is not so. The inability of GS-GOGAT pathway to assimilate all the nitrogenase-derived ammonia, despite their comparable levels, can be explained as follows:

1. The heterocysts which are the sites of nitrogen fixation are only a small portion of the total cell population. Considering that nitrogenase is located only in heterocysts, while GS is located both in heterocysts and vegetative cells, the total GS activity in heterocysts would be only a small percentage of that observed in whole filaments of the cyanobiont and would be far less than the total nitrogenase activity.
2. GS would be required not only to assimilate newly fixed ammonia but also the ammonia produced during protein turnover.
3. As in *Azolla*,[217] there is no strict linear relationship between GS and nitrogenase throughout the lichen thallus. Maximum nitrogenase activity has generally been found in the central portion of the thallus, whereas GS activity is greater toward the apex.[115,172]

Taken together, the above observations indicate inability of the cyanobiont GS to assimilate all the ammonia produced during nitrogen fixation. This leads to ammonia being liberated. Such a situation, when created in free-living cyanobacteria by inhibiting GS using MSX, also leads to liberation of ammonia produced during nitrogen fixation.[162]

Ammonium transport in cyanobacteria occurs in response to the electrical potential across the membrane.[165] It has been suggested that a decrease in the transmembrane electrical potential, by compounds like colicin K,[218] should lead to a considerable loss of ammonia from the cyanobiont cells in symbiosis; such a compound may be produced by the fungus.[164] However, one must remember that membrane potential is involved in several processes necessary for cellular metabolism, including nitrogenase,[119-222] and a general lowering of membrane potential may not be desirable for the cyanobiont. In addition, cyanobionts in other symbioses have ammonium transport systems intact and there is no indication of their membrane potential being affected.

UTILIZATION OF LIBERATED FIXED N BY OTHER SYMBIONTS (ROUTES OF N METABOLISM)

Pathways of nitrogen metabolism in *P. canina* and *P. aphthosa* have been studied using $^{15}N_2$.[78,146,147] Studies of Rai et al.[147] have shown that in *P. canina* the ammonia retained by the cyanobiont (44% of the fixed N) is assimilated via GS-GOGAT, and that ammonia liberated from the cyanobiont (55% of the fixed N) is assimilated via GDH in the mycobiont. In *P. aphthosa*,[78,146,159] only about 5% of the ammonia produced during nitrogen fixation is assimilated by the cyanobiont via GS-GOGAT pathway, while the rest is transferred to the mycobiont. This ammonia is assimilated via GDH in the cephalodial mycobiont. The glutamate thus produced is converted to various amino acids through the action of aminotransferases. Alanine has been suggested to be the compound transferred from cephalodia to the main thallus. Based on the ^{15}N-kinetics studies, an outline of the route of nitrogen metabolism in *P. aphthosa* is given in Figure 2.

FUTURE PROSPECTS

A considerable amount of new information has recently been presented regarding the physiology and biochemistry of cyanolichens. However, several key questions are still unanswered. The areas of these questions include:

1. Mechanism of repression of GS in the cyanobiont
2. Mechanism of the modification of the carbon metabolism in the cyanobiont and the mechanism of carbohydrate translocation
3. Mechanism of increase in heterocyst frequency of the cyanobionts in tripartite lichens

Information on the above points would help in the manipulation of free-living cyanobacteria for enhanced nitrogen fixation and ammonia production.[223,224] Such modified strains can be used directly in the crop fields or in laboratories for photobiological production of ammonia.

Nitrogen fixation is the key process in utilization of atmospheric molecular nitrogen, but this capability is limited only to prokaryotes.[225-227] (However, see Yamada and Sakaguchi[228] and Weathers et al.[229]). N_2-fixing cyanobacteria form symbioses with a wide range of plants, but none with crop plants.[174,230] Establishment of artificial symbioses between nitrogen-fixing cyanobacteria and economically important plants would be highly desirable since it would reduce the cost of production by reducing/eliminating the need for nitrogenous fertilizers. Although early attempts in this direction have been unsuccessful, with newer information, further attempts would be worthwhile.[231-233] Indeed, encouraging results have been obtained recently (see Chapter 8).

Several events during early stages of lichen development can be studied in the laboratory if lichen symbioses can be created there. This has been successfully achieved in lichens containing green algae.[234-236] However, cyanolichens have not yet been tried. This aspect requires more attention than has been given up to this time.

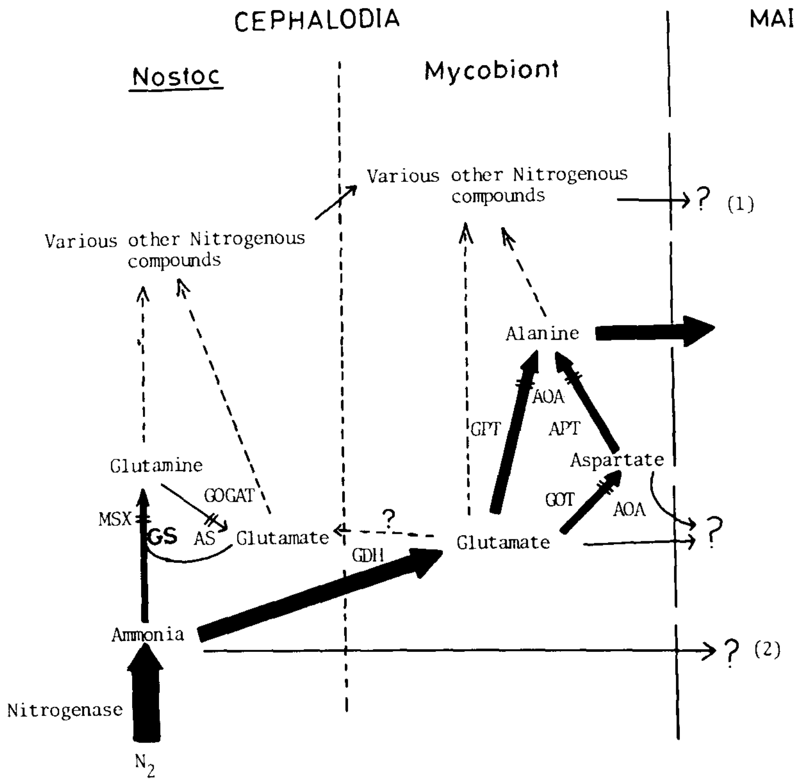
CEPHALODIA
MAIN THALLUS
Nostoc
Mycobiont
Various other Nitrogenous compounds
? (1)
Various other Nitrogenous compounds
Alanine
AOA
GPT
APT
Aspartate
Glutamine
GOGAT
GOT
AOA
MSX
GS
AS
Glutamate
?
GDH
Glutamate
?
Ammonia
? (2)
Nitrogenase
N_2

FIGURE 2. Routes of nitrogen metabolism in cephalodia of *Peltigera aphthosa* (based on ^{15}N kinetics and enzymic studies).[78] Bold arrows = major routes; thin arrows = minor routes; broken arrows = possible minor routes (not studied in detail); ? indicates possible transfer compounds suggested by others.[115,213]

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REFERENCES

1. **Ahmadjian, V. and Paracer, S.,** *Symbiosis,* University Press of New England, Hanover, NH, 1986, 90.
2. **Galun, M. and Bubrick, P.,** Physiological interactions between the partners of lichen symbiosis, in *Encyclopedia of Plant Physiology, Vol. 17, Cellular Interactions,* Linskens, H. F. and Heslop-Harrison, J., Eds., Springer-Verlag, Berlin, 1984, 362.
3. **Stewart, W. D. P., Rowell, P., and Rai, A. N.,** Symbiotic nitrogen-fixing cyanobacteria, in *Nitrogen Fixation,* Stewart, W. D. P. and Gallon, J. R., Eds., Academic Press, London, 1980, 239.
4. **Smith, D. C. and Douglas, A. E.,** *The Biology of Symbiosis,* Edward Arnold, London, 1987, 113.
5. **Galun, M.,** *Handbook of Lichenology, Vol. 1—3,* CRC Press, Boca Raton, FL, 1988.
6. **Knapp, E.,** Über *Geosiphon pyriforme* Fr. Wettst., eine intrazelluläre Pilz-Algen Symbiose, *Ber. Dtsch. Bot. Ges.,* 51, 210, 1933.
7. **Schnepf, E.,** Zur Feinstruktur von *Geosiphon pyriforme,* ein Versuch zur Deutung cytoplasmatischer Membranen und Kompartimente, *Arch. Mikrobiol.,* 49, 112, 1964.
8. **Marton, K. and Galun, M.,** *In vitro* dissociation and reassociation of the symbionts in the lichen *Heppia echinulata, Protoplasm,* 87, 135, 1976.

9. **Ahmadjian, V.,** A guide to the algae occurring as lichen symbionts: isolation, culture, cultural physiology and identification, *Phycologia,* 6, 127, 1967.
10. **Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y.,** Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J. Gen. Microbiol.,* 111, 1, 1979.
11. **Ahmadjian, V.,** Lichens, in *Physiology and Biochemistry of Algae,* Lewin, R. A., Ed., Academic Press, New York, 1962, 817.
12. **Bornet, E.,** Recherches sur les gonidies des lichens, *Ann. Sci. Nat. Bot., (Ser. 5),* 17, 145, 1873.
13. **Galloe, O.,** *Natural History of the Danish Lichens, Part 6,* Munksgaard, Copenhagen, 1939.
14. **Henssen, A.,** Eine Revision der Flechtenfamilien Lichinaceae und Ephebaceae, *Symb. Bot. Ups.,* 18(1), 123, 1967.
15. **Geitler, L.,** Beiträge zur Kenntnis der Flechtensymbiose IV—V, *Arch. Protistenkd.,* 82, 51, 1934.
16. **Degelius, G.,** The lichen genus *Collema* in Europe: morphology, taxonomy, ecology, *Symb. Bot. Ups.,* 13(2), 499, 1954.
17. **Linkola, K.,** Kulturen mit *Nostoc*-Gonidien der *Peltigera*-Arten, *Ann. Zool. Soc. Zool. Bot. Fenn. Vanamo,* 1, 1, 1923.
18. **Watanabe, A. and Kiyohara, T.,** Symbiotic blue-green algae in lichens, liverworts and cycads, in *Studies on Microalgae and Photosynthetic Bacteria,* Japanese Society of Plant Physiologists, Tokyo, 1963, 189.
19. **Schiman, H.,** Beiträge zur Lebelsgeschichte homoeomerer und heteromerer Cyanophyceen-Flechten, *Österr. Bot. Z.,* 104, 409, 1957.
20. **Kele, R. A.,** Isolation of the Mycobiont and Phycobiont of an Underwater Lichen, *Hydrothyrea venosa,* Honors thesis, Biology Department, Clark University, Worcester, MA, 1964.
21. **Danilov, A. V.,** Le *Nostoc* en état de symbiose, *Russ. Arkh. Protist.,* 6, 83, 1927.
22. **Lamb, I. M.,** On the morphology, physiology, and taxonomy of the genus *Stereocaulon, Can. J. Bot.,* 29, 522, 1951.
23. **Santesson, R.,** Foliicolous Lichens. I, *Symb. Bot. Ups.,* 12, 590, 1952.
24. **Henssen, A.,** Was ist *Pyrenothrix nigra?, Ber. Dtsch. Bot. Ges.,* 77, 317, 1964.
25. **Duvigneaud, P.,** Les *Stereocaulon* des hautes montagnes du Kivu, *Lejeunia Mém.,* 14, 144, 1955.
26. **Hitch, C. J. B. and Millbank, J. W.,** Nitrogen metabolism in lichens. VII. Nitrogenase activity and heterocyst frequency in lichens with blue-green phycobionts, *New Phytol.,* 75, 239, 1975.
27. **Huss-Danell, K.,** The cephalodia and their nitrogenase activity in the lichen *Stereocaulon paschale, Z. Pflanzenphysiol.,* 95, 431, 1979.
28. **Geitler, L.,** Zur Kenntnis der Flechte *Thermutis velutina, Österr. Bot. Z.,* 112, 263, 1965.
29. **Swinscow, T. D. V.,** The marine species of *Arthopyrenia* in the British Isles, *Lichenologist,* 3, 55, 1965.
30. **Jaag, O.,** Untersuchungen über die Vegetation und Biologie der Algen des nackten Gesteins in den Alpen, im Jura und im schweizerrischen Mitteland, *Beitr. Kryptogamenflora Schweiz,* 9, 3, 1945.
31. **Geitler, L.,** Beiträge zur Kenntnis der Flechtensymbiose. VI, *Arch. Protistenkd.,* 88, 161, 1937.
32. **Bubrick, P. and Galun, M.,** Cyanobiont diversity in the Lichinaceae and Heppieaceae, *Lichenologist,* 16, 279, 1984.
33. **Jahns, H. M.,** Anatomy, morphology, and development, in *The Lichens,* Ahmadjian, V. and Hale, M. E., Eds., Academic Press, New York, 1973, 3.
34. **Peveling, E.,** Die Darstellung der Oberflächenstrukturen von Flachten mit dem Raster-Elektronenmikroskop, *Ber. Dtsch. Bot. Ges.,* 4, 89, 1970.
35. **Forssell, K. B. J.,** Studier öfver cephalodierna, *K. Sven. Vetenskapsatad,* 8, 1, 1883.
36. **Jordan, W. P.,** The internal cephalodia of the genus *Lobaria, Bryologist,* 73, 669, 1970.
37. **Jordan, W. P. and Rickson, R.,** Cyanophyte cephalodia in the lichen genus *Nephroma, Am. J. Bot.,* 58, 562, 1971.
38. **Moreau, F.,** Les Lichens, in *Encyclopedie Biologie,* Vol. 2, Lechevalier, Paris, 1928, 1.
39. **Brodo, I. M. and Richardson, D. H. S.,** Chimeroid associations in the genus *Peltigera, Lichenologist,* 10, 157, 1978.
40. **James, P. W. and Henssen, A.,** The morphological and taxonomic significance of cephalodia, in *Lichenology: Progress and Problems,* Brown, D. H., Hawksworth, D. L., and Bailey, R. H., Eds., Academic Press, London, 1976, 22.
41. **Poelt, J.,** Classification, in *The Lichens,* Ahmadjian, V. and Hale, M. E., Eds., Academic Press, New York, 1973, 599.
42. **Poelt, J.,** Systematic evaluation of morphological characters, in *The Lichens,* Ahmadjian, V. and Hale, M. E., Eds., Academic Press, New York, 1973, 91.
43. **Henssen, A.,** *Thyrea radiata,* eine *Lempholemma*-Art mit Hormocystangien, *Ber. Dtsch. Bot. Ges.,* 81, 176, 1968.
44. **Henssen, A.,** An interesting new species of *Lempholemma* from Canada, *Lichenologist,* 4, 99, 1969.
45. **Bitter, G.,** Zur Morphologie und Systematik von *Parmelia,* Untergattung *Hypogymnia, Hedwigia,* 40, 171, 1901.
46. **Du Rietz, G. E.,** Die Soredien und Isidien du Flechten, *Sven. Bot. Tidskr.,* 18, 371, 1924.

47. **Frey, E.**, Beiträge zu einer Lichenenflora der Schwiez II und III. Die Familie Physciaceae, *Ber. Schweiz. Bot. Ges.*, 73, 389, 1963.
48. **Darbishire, O. V.**, Die deutschen Pertusariaceen mit besonderer Berusksichtigung ihrer Soredienbildung, *Bot. Jahrb.*, 22, 593, 1897.
49. **Barkman, J. J.**, *Phytosociology and Ecology of Cryptogamic Epiphytes,* Van Gorcum, Assen, Netherlands, 1958.
50. **Hale, M. E.**, *Lichen Handbook,* Smithsonian Institute, Washington, D.C., 1961.
51. **Bailey, R. H.**, Ecological aspects of dispersal and establishment in lichens, in *Lichenology: Progress and Problems,* Brown, D. H., Hawksworth, D. L., and Bailey, R. H., Eds., Academic Press, London, 1976, 215.
52. **des Abbayes, H.**, *Traite de Lichenologie,* Lechevalier, Paris, 1951.
53. **Gregory, P. H.**, *Microbiology of the Atmosphere,* 2nd ed., Leonard Hill, London, 1973.
54. **Smith, D. C.**, The biology of lichen thalli, *Biol. Rev.*, 37, 537, 1962.
55. **Haynes, F. N.**, Lichens, *Viewpoints Biol.*, 3, 64, 1964.
56. **Bailey, R. H.**, Studies on the dispersal of lichen soredia, *J. Linn. Soc. London Bot.*, 59, 479, 1966.
57. **Brodie, H. J. and Gregory, P. H.**, The action of wind in the dispersal of spores from cup-shaped plant structures, *Can. J. Bot.*, 31, 402, 1953.
58. **Brodie, H. J.**, The splash cup dispersal mechanism in plants, *Can. J. Bot.*, 29, 224, 1951.
59. **Bailey, R. H.**, Dispersal of lichen soredia in water trickles, *Rev. Bryol. Lichenol.*, 36, 314, 1968.
60. **Durrell, L. W.**, An electron microscope study of algal hyphal contact in lichens, *Mycopathol. Mycol. Appl.*, 31, 273, 1967.
61. **Peat, A.**, Fine structure of the vegetative thallus of the lichen *Peltigera polydactyla, Arch. Mikrobiol.*, 61, 212, 1968.
62. **Peveling, E.**, Das Vorkommen von Starke in Chlorophyceen-Phycobionten, *Planta,* 93, 82, 1970.
63. **Peveling, E.**, Investigations into the ultrastructure of lichens, in *Lichenology: Progress and Problems,* Brown, D. H., Hawksworth, D. L., and Bailey, R. H., Eds., Academic Press, London, 1976, 17.
64. **Honegger, R.**, Cytological aspects of the mycobiont-phycobiont relationships in lichens, *Lichenologist,* 16, 111, 1984.
65. **Honegger, R.**, Fine structure of different types of symbiotic relationships in lichens, in *Lichen Physiology and Cell Biology,* Brown, D. H., Ed., Plenum Press, New York, 1985, 287.
66. **Boissiere, M. C.**, Cytochemical ultrastructure of *Peltigera canina.* Some features related to its symbiosis, *Lichenologist,* 14, 1, 1982.
67. **Paran, N., Ben-Shaul, Y., and Galun, M.**, Fine structure of the blue-green phycobiont and its relation to the mycobiont in two *Gonohymenia* lichens, *Arch. Mikrobiol.*, 76, 103, 1971.
68. **Roskin, P. A.**, Ultrastructure of the host-parasite interaction in the basidiolichen *Cora pavonia* (Web.) E. Fries, *Arch. Mikrobiol.*, 70, 183, 1970.
69. **Bergman, B. and Hallbom, L.**, *Nostoc* of *Peltigera canina* when lichenized and isolated, *Can. J. Bot.*, 60, 2092, 1982.
70. **Koriem, A. M. and Ahmadjian, V.**, An ultrastructural study of lichenized and cultured *Nostoc* phycobionts of *Peltigera canina, Peltigera rufescens,* and *Peltigera spuria, Endocytol. Cell Res.*, 3, 65, 1986.
71. **Bousfield, J. and Peat, A.**, The ultrastructure of *Collema tenax* with particular reference to microtubule like inclusions and vesicle production by the phycobiont, *New Phytol.*, 76, 121, 1976.
72. **Spector, D. L. and Jensen, T. E.**, Fine structure of *Leptogium cyanescens* and its cultured phycobiont *Nostoc commune, Bryologist,* 80, 445, 1977.
73. **Boissiere, M. C.**, Mise en évidence cytochimique en microscopie électronique de polyglucosides de réserve chez des *Nostoc* libres et lichénisés, *C. R. Acad. Sci. Paris Ser. D,* 274, 2643, 1972.
74. **Jacobs, J. B. and Ahmadjian, V.**, The ultrastructure of lichens. V. *Hydrothyrea venosa,* a fresh water lichen, *New Phytol.*, 72, 155, 1973.
75. **Rai, A. N., Rowell, P., and Stewart, W. D. P.**, Nitrogenase activity and dark CO_2 fixation in the lichen *Peltigera aphthosa* Willd., *Planta,* 151, 256, 1981.
76. **Bergman, B. and Huss-Danell, K.**, Ultrastructure of *Stigonema* in the cephalodia of *Stereocaulon paschale, Lichenologist,* 15, 181, 1983.
77. **Peveling, E.**, Electronenoptische Untersuchungen an Flechten. IV. Die Feinstruktur einiger Flechten mit Cyanophyceen-Phycobionten, *Protoplasma,* 68, 209, 1969.
78. **Rai, A. N.**, Studies on the Nitrogen-Fixing Lichen *Peltigera aphthosa* Willd., Ph.D. thesis, Dundee University, Dundee, U.K., 1980.
79. **Stewart, W. D. P., Rai, A. N., Reed, R. H., Creach, E., Codd, G. A., and Rowell, P.**, Studies on the N_2-fixing lichen *Peltigera aphthosa,* in *Current Perspectives in Nitrogen Fixation,* Gibson, A. H. and Newton, W. E., Eds., Australian Academy of Sciences, Canberra, 1981, 237.
80. **Czeczuga, B.**, Studies of phycobiliproteins in algae. III. Phycobiliproteins in the phycobionts of the *Peltigera* species, *Nova Hedwigia,* 36, 687, 1982.

81. **Peveling, E.,** Electronenoptische Untersuchungen an Flechten. III. Cytologische Differenzierungen der Pilzzellen in Zusammenhang mit ihrer symbiontischen Lebensweise, *Z. Pflanzenphysiol.*, 61, 151, 1969.
82. **Peveling, E.,** Vesicles in the phycobiont sheath as possible transfer structures between the symbionts in the lichen *Lichina pygmea, New Phytol.*, 72, 343, 1973.
83. **Boissiere, J. C. and Boissiere, M. C.,** Etude ultrastructurale et cytochimique de la paroi de *Nostoc* libre et lichenisés, *C.R. Acad. Sci. Paris Ser. D*, 278, 2767, 1974.
84. **Honegger, R.,** Cytological aspects of the triple symbiosis in *Peltigera aphthosa, J. Hattori Bot. Lab*, 52, 379, 1982.
85. **Moore, R. T. and McAlear, J. H.,** Fine structure of mycota, *Mycologia*, 52, 805, 1960.
86. **Boissiere, J. C.,** Présence d'haustoriums chez le lichen *Lichina pygmea* (Light) A.C. Ag. et role de la paroi des gonidies dans le contact entre les symbiontes des lichens à cyanophytes, *Rev. Bryol. Lichenol.*, 43, 176, 1977.
87. **Galun, M., Paran, N., and Ben-Shaul, Y.,** Structural modifications of the phycobiont in the lichen thallus, *Protoplasma*, 69, 85, 1970.
88. **Chervin, R. E., Baker, G. E., and Hohl, H. R.,** The ultrastructure of phycobiont and mycobiont in the two species of *Usnea, Can. J. Bot.*, 46, 241, 1968.
89. **Galun, M., Ben-Shaul, Y., and Paran, N.,** Fungus-alga association in lichens of the Teloschistaceae: an ultrastructural study, *New Phytol.*, 70, 837, 1971.
90. **Collins, C. R. and Farrar, J. F.,** Structural resistance to mass transfer in the lichen *Xanthoria parietina, New Phytol.*, 81, 71, 1978.
91. **Dazzo, F. B. and Truchet, G. L.,** Interactions of lectins and their saccharide receptors in the *Rhizobium*-legume symbiosis, *J. Membr. Biol.*, 73, 1, 1983.
92. **Paau, A. S., Leps, W. T., and Brill, W. J.,** Agglutinin from alfalfa necessary for binding and nodulation by *Rhizobium meliloti, Science*, 213, 1513, 1981.
93. **Hardman, J. T., Hale, M. E., Hardman, P. K., and Beck, M. L.,** Report on activity of lichen lectins, *Lichenologists*, 15, 303, 1983.
94. **Lockhart, C. M., Rowell, P., and Stewart, W. D. P.,** Phytohaemagglutinins from the nitrogen-fixing lichens *Peltigera canina* and *Peltigera polydactyla, FEMS Microbiol. Lett.*, 3, 127, 1978.
95. **Petit, P., Lallemant, R., and Savoye, D.,** Purified phytolectin from the lichen *Peltigera canina var. canina* which binds to the phycobiont cell walls and its use as cytochemical marker *in situ, New Phytol.*, 94, 103, 1983.
96. **Barret, J. T. and Howe, M. L.,** Hemagglutination and hemolysis by lichen extracts, *Appl. Microbiol.*, 16, 1137, 1968.
97. **Bubrick, P. and Galun, M.,** unpublished results (quoted in Reference 2).
98. **Rai, A. N.,** unpublished results, 1980.
99. **Petit, P.,** Phytolectins from the nitrogen-fixing lichen *Peltigera horizontalis:* the binding pattern of primary protein extract, *New Phytol.*, 91, 705, 1982.
100. **Lallemant, R. and Savoye, D.,** Lectins and morphogenesis: facts and outlooks, in *Lichen Physiology and Cell Biology*, Brown, D. H., Ed., Plenum Press, New York, 1985, 335.
101. **Mellor, R. B., Gadd, G. M., Rowell, P., and Stewart, W. D. P.,** A phytohaemagglutinin from the *Azolla-Anabaena* symbiosis, *Biochem. Biophys. Res. Commun.*, 99, 1348, 1981.
102. **Mellor, R. B., Rowell, P., and Stewart, W. D. P.,** The non-random distribution of lectin in the *Azolla caroliniana-Anabaena azollae* symbiosis, in *Lectins — Biology, Biochemistry, Clinical Biochemistry*, Vol. 2, Bog-Hansen, T. G., Ed., Walter de Gruyter, Berlin, 1982, 105.
103. **Dick, H. and Stewart, W. D. P.,** The occurrence of fimbriae on a N_2-fixing cyanobacterium which occurs in lichen symbiosis, *Arch. Microbiol.*, 124, 107, 1980.
104. **Kershaw, K. A.,** *Physiological Ecology of Lichens*, Cambridge University Press, Cambridge, 1985.
105. **Drew, E. A.,** Some Aspects of Carbohydrate Metabolism in Lichens, D. Phil. thesis, University of Oxford, Oxford, 1966.
106. **Feige, B.,** Stoffwechselphysiologische Untersuchungen an der tropischen Basidiolichene *Cora pavonia* (Sw.) Fr., *Flora (Jena)*, 160A, 169, 1969.
107. **Drew, E. A. and Smith, D. C.,** Studies in the physiology of lichens. VII. The physiology of *Nostoc* symbiont of *Peltigera polydactyla* compared with cultured and free-living forms, New Phytol., 66, 379, 1967.
108. **Farrar, J. F.,** Lichen physiology: progress and pitfalls, in *Air Pollution and Lichens*, Ferry, B. W., Baddeley, M. S., and Hawksworth, D. L., Eds., Athlone Press, London, 1973, 238.
109. **Kershaw, K. A., Morris, T., Tysiaczny, M. J., and MacFarlane, J. D.,** Physiological-environmental interactions in lichens. VIII. The environmental control of dark CO_2 fixation in *Parmelia caperata* (L.) Ach. and *Peltigera canina* var. *praetextata* Hue, *New Phytol.*, 83, 433, 1979.
110. **Smith, D. C., Muscatine, L., and Lewis, D. H.,** Carbohydrate movement from autotrophs to heterotrophs in parasitic and mutualistic symbioses, *Biol. Rev.*, 44, 17, 1969.

111. **Drew, E. A. and Smith, D. C.**, Studies in the physiology of lichens. VIII. Movement of glucose from alga to fungus during photosynthesis in the thallus of *Peltigera polydactyla, New Phytol.*, 66, 389, 1967.
112. **Richardson, D. H. S., Hill, D. J., and Smith, D. C.**, Lichen physiology. XI. The role of the alga in determining the pattern of carbohydrate movement between lichen symbionts, *New Phytol.*, 67, 469, 1968.
113. **Drew, E. A. and Smith, D. C.**, The physiology of the symbiosis in *Peltigera polydactyla* (Neck.) Hoffm., *Lichenologist*, 3, 197, 1966.
114. **Richardson, D. H. S., Smith, D. C., and Lewis, D. H.**, Carbohydrate movement between symbionts of lichens, *Nature (London)*, 214, 879, 1967.
115. **Englund, B.**, The physiology of the lichen *Peltigera aphthosa*, with special reference to the blue-green phycobiont (*Nostoc* sp.), *Physiol. Plant.*, 41, 298, 1977.
116. **Feige, G. B.**, Untersuchungen zur Physiologie der Cephalodien der Flechte *Peltigera aphthosa* (L.) Willd. I. Die photosynthetische ^{14}C-Markierung der Lipidfraktion, *Z. Pflanzenphysiol.*, 80, 377, 1976.
117. **Feige, G. B.**, Untersuchungen zur Physiologie der Cephalodien der Flechte *Peltigera aphthosa* (L.) Willd. II. Das photosynthetische ^{14}C-Markierungsmuster und der Kohlenhydrattransfer zwischen Phycobiont und Mycobiont, *Z. Pflanzenphysiol.*, 80, 386, 1976.
118. **Smith, D. C.**, The role of nutrient exchange in recognition between symbionts, *Ber. Dtsch. Bot. Ges.*, 94, 517, 1981.
119. **Smith, D. C.**, Transport from symbiotic algae and symbiotic chloroplasts to host cells, *Symp. Soc. Exp. Biol.*, 28, 485, 1974.
120. **Smith, D. C.**, Mechanisms of nutrient movement between lichen symbionts, in *Cellular Interactions in Symbiosis and Parasitism*, Cook, C. B., Pappas, P. W., and Rudolph, E. D., Eds., Ohio State University Press, Columbus, 1980, 197.
121. **Smith, D. C.**, Is a lichen a good model for biological interactions in nutritional-limited environments?, in *Strategies for Microbial Life in Extreme Environments*, Shilo, M., Ed., Dahlem Konferenzen, Berlin, 1979, 291.
122. **Cowan, D. A., Green, T. G. A., and Wilson, A. T.**, Lichen metabolism. I. The use of tritium-labelled water in studies of anhydrobiotic metabolism in *Ramalina celestri* and *Peltigera polydactyla, New Phytol.*, 82, 489, 1979.
123. **Hill, D. J.**, The movement of carbohydrate from the alga to the fungus in the lichen *Peltigera polydactyla, New Phytol.*, 71, 31, 1972.
124. **Peveling, E. and Hill, D. J.**, The localization of an insoluble intermediate in glucose production in the lichen *Peltigera polydactyla, New Phytol.*, 73, 767, 1974.
125. **Smith, D. C.**, Symbiosis and the biology of lichenized fungi, *Symp. Soc. Exp. Biol.*, 29, 373, 1975.
126. **Collins, C. R. and Farrar, J. F.**, Structural resistance to mass transfer in the lichen *Xanthoria parietina, New Phytol:*, 81, 71, 1978.
127. **Tapper, R. C.**, *Studies on Carbohydrate Efflux from Lichen Algae*, Ph.D. thesis, University of Bristol, Bristol, U.K., 1979.
128. **Boissiere, M. C.**, Activité phosphatasique neutre chez le phycobionte de *Peltigera canina* comparée à celle d'un *Nostoc* libre, *C.R. Acad. Sci. Paris Ser. D*, 277, 1649, 1973.
129. **Chambers, S., Morris, M., and Smith, D. C.**, Lichen physiology. XV. The effect of digitonin and other treatments on biotrophic transport of glucose from alga to fungus in *Peltigera polydactyla, New Phytol.*, 76, 485, 1976.
130. **Smith, D. C.**, The physiology of *Peltigera polydactyla* (Neck.) Hoffm., *Lichenologist*, 1, 209, 1961.
131. **Millbank, J. W. and Kershaw, K. A.**, Nitrogen metabolism in lichens. I. Nitrogen fixation in the cephalodia of *Peltigera aphthosa, New Phytol.*, 68, 721, 1969.
132. **Hill, D. J. and Smith, D. C.**, Lichen physiology. XII. The inhibition technique, *New Phytol.*, 71, 15, 1972.
133. **Richardson, D. H. S.**, The surface physiology of lichens with particular reference to carbohydrate transfer between the symbionts, in *Surface Physiology of Lichens*, Vicente, C., Brown, D. H., and Legaz, H. E., Eds., Universidad Complutense de Madrid, Madrid, 1985, 25.
134. **Goas, G. and Bernard, T.**, Contribution a l'étude du metabolisme azote des lichens; les différentes formes dázote de quelques espèćés de la famille des Stictacées, *C.R. Acad. Sci. Paris Ser. D*, 265, 1187, 1967.
135. **Green, T. G. A., Horstmann, J., Bonnett, H., Wilins, A., and Silvester, W. B.**, Nitrogen fixation by members of the Stictaceae (Lichenes) of New Zealand, *New Phytol.*, 84, 339, 1980.
136. **Hitch, C. J. B. and Stewart, W. D. P.**, Nitrogen fixation by lichens in Scotland, *New Phytol.*, 72, 509, 1973.
137. **Millbank, J. W.**, Nitrogen metabolism in lichens. IV. The nitrogenase activity of the *Nostoc* phycobiant in *Peltigera canina, New Phytol.*, 71, 1, 1972.
138. **Scott, G. D.**, Further investigations of some lichens for nitrogen fixation, *New Phytol.*, 55, 111, 1956.
139. **Smith, D. C.**, Studies in the physiology of lichens. I. The effects of starvation and of ammonia absorption upon the nitrogen content of *Peltigera polydactyla, Ann. Bot.*, 24, 52, 1960.
140. **Sampaio, M. J. A. M., Rai, A. N., Rowell, P., and Stewart, W. D. P.**, Occurrence, synthesis and activity of glutamine synthetase in N_2-fixing lichens, *FEMS Microbiol. Lett.*, 6, 107, 1979.

141. **Ramakrishnan, S. and Subramanian, S. S.,** Amino acids of *Peltigera canina, Curr. Sci.*, 33, 522, 1964.
142. **Ramakrishnan, S. and Subramanian, S. S.,** Amino acids of *Cladonia rangiferina, Cladonia gracilis* and *Lobaria isidiosa, Curr. Sci.*, 34, 345, 1965.
143. **Ramakrishnan, S. and Subramanian, S. S.,** Amino acids of *Lobaria subisidiosa, Umbilicaria pustulata, Parmelia nepalensis* and *Ramelina sinensis, Curr. Sci.*, 35, 124, 1966.
144. **Solberg, Y. J.,** Studies on the chemistry of lichens. VIII. An examination of the free sugars and ninhydrin positive compounds of several Norwegian lichen species, *Lichenologist*, 4, 271, 1970.
145. **Solberg, Y. J.,** Studies on the chemistry of lichens. XIV. Chemical investigations of the lichen species *Anaptychia fusca, Peltigera canina* and *Omphalodiscus spodochrous, Z. Naturforsch.*, 30c, 445, 1975.
146. **Rai, A. N., Rowell, P., and Stewart, W. D. P.,** $^{15}N_2$-Incorporation and metabolism in the lichen *Peltigera aphthosa* Willd., *Planta*, 152, 544, 1981.
147. **Rai, A. N., Rowell, P., and Stewart, W. D. P.,** Interactions between cyanobacterium and fungus during $^{15}N_2$-incorporation and metabolism in the lichen *Peltigera canina, Arch. Microbiol.*, 134, 136, 1983.
148. **Bernard, T. and Goas, G.,** Contribution à l'étude du metabolisme azote des lichens. Characterisation et dosages des methylamines de quelques espèces de la famille des Stictacées, *C.R. Acad. Sci. Paris (Ser. D)*, 267, 622, 1968.
149. **Bernard, T. and Larher, F.,** Contribution à l'étude du metabolisme azote des lichens. Role de la glycine $^{14}C_2$ dans la formation des methylamines chez *Lobaria laetevirens, C.R. Acad. Sci. Paris (Ser. D)*, 272, 568, 1971.
150. **Smith, T. A.,** Putrescine, spermidine and spermine in higher plants, *Phytochemistry*, 9, 1479, 1970.
151. **Cohen, S. S.,** *Introduction to the Polyamines*, Prentice-Hall, Englewood Cliffs, N.J., 1971.
152. **Smith, T. A.,** Recent advances in the biochemistry of plant amines, *Phytochemistry*, 14, 865, 1975.
153. **Lange, O. L.,** Versuche zur Hitze-und Trochenresistenz der Flechten, *Flora (Jena)*, 140, 39, 1953.
154. **Lange, O. L.,** Die funktionellen Anpassungen der Flechten an die ökologischen Bedingungen arider Gebiete, *Ber. Dtsch. Bot. Ges.*, 82, 3, 1969.
155. **Hallbom, L.,** Sarcosine: a possible regulatory compound in the *Peltigera praetextata-Nostoc* symbiosis, *FEMS Microbiol. Lett.*, 22, 119, 1984.
156. **Bernard, T. and Goas, G.,** Biosynthèse de la sticticin chez le lichen *Lobaria laetevirens, Physiol. Plant.*, 53, 71, 1981.
157. **Cohen-Bazire, G. and Bryant, D. A.,** Phycobilisomes: composition and structure, in *The Biology of Cyanobacteria*, Carr, N. G. and Whitton, B. A., Eds., Blackwell Scientific, Oxford, 1982, 143.
158. **Solomon, H.,** Über das Vorkommen und die Aufnahme einiger wichtiger N'ahrsalze bei den Flechten, *Jahrb. Wiss. Bot.*, 54, 309, 1914.
159. **Rai, A. N., Rowell, P., and Stewart, W. D. P.,** NH_4^+ assimilation and nitrogenase regulation in the lichen *Peltigera aphthosa* Willd., *New Phytol.*, 85, 545, 1980.
160. **Rai, A. N.,** Nitrogen metabolism, in *Lichenology*, Vol. 1, Galun, M., Ed., CRC Press, Boca Raton, FL, 1988, 201.
161. **Dharmawardene, M. W. N., Haystead, A., and Stewart, W. D. P.,** Glutamine synthetase of the nitrogen-fixing alga *Anabaena cylindrica, Arch. Microbiol.*, 90, 281, 1973.
162. **Stewart, W. D. P. and Rowell, P.,** Effects of *L*-methionine-DL-suphoximine on the assimilation of newly fixed NH_3, acetylene reduction and heterocyst production in *Anabaena cylindrica, Biochem. Biophys. Res. Commun.*, 65, 846, 1975.
163. **Wolk, C. P., Thomas, J., Shaffer, P. W., Austin, S. M., and Galonsky, A.,** Pathways of nitrogen metabolism after fixation of ^{13}N-labelled nitrogen gas by the cyanobacterium *Anabaena cylindrica, J. Biol. Chem.*, 251, 5027, 1979.
164. **Kleiner, D., Phillips, S., and Fitzke, E.,** Pathways and regulatory aspects of N_2 and NH_4^+ assimilation in N_2-fixing bacteria, in *Biology of Inorganic Nitrogen and Sulfur*, Bothe, H. and Trebst, A., Eds., Springer-Verlag, Berlin, 1981, 131.
165. **Rai, A. N., Rowell, P., and Stewart, W. D. P.,** Evidence for an ammonium transport system in free-living and symbiotic cyanobacteria, *Arch. Microbiol.*, 137, 241, 1984.
166. **Rai, A. N., Lindblad, P., and Bergman, B.,** Absence of the glutamine synthetase-linked methylammonium (ammonium) transport system in the cyanobiont of *Cycas*-cyanobacterial symbiosis, *Planta*, 169, 379, 1986.
167. **Smith, D. C.,** Studies in the physiology of lichens. II. Absorption and utilization of some simple organic nitrogen compounds by *Peltigera polydactyla, Ann. Bot.*, 24, 172, 1960.
168. **Smith, D. C.,** Studies in the physiology of lichens. III. Experiments with dissected discs of *Peltigera polydactyla, Ann. Bot.*, 24, 188, 1960.
169. **Shapiro, I. A.,** Urease activity in lichens, *Fiziol. Rast.*, 24, 1135, 1977.
170. **Stewart, W. D. P. and Rowell, P.,** Modifications of nitrogen-fixing algae in lichen symbioses, *Nature (London)*, 265, 371, 1977.
171. **Bernard, T. and Goas, G.,** Glutamate dehydrogenases du lichen *Lobaria laetevirens* (Lightf.) Zahlbr. caractéristiques de l'enzyme du Champignan, *Physiol. Veg.*, 17, 535, 1979.

172. **Rowell, P., Rai, A. N., and Stewart, W. D. P.**, Studies on the nitrogen metabolism of the lichens *Peltigera aphthosa* and *Peltigera canina*, in *Lichen Physiology and Cell Biology*, Brown, D. H., Ed., Plenum Press, New York, 1985, 145.

173. **Rai, A. N., Rowell, P., and Stewart, W. D. P.**, Glutamate synthase activity in symbiotic cyanobacteria, *J. Gen. Microbiol.*, 126, 515, 1981.

174. **Stewart, W. D. P., Rowell, P., and Rai, A. N.**, Cyanobacteria-eukaryotic plant symbioses, *Ann. Microbiol. (Inst. Pasteur)*, 134B, 205, 1983.

175. **Hallbom, L., Bergman, B., and Rai, A. N.**, Immunogold localization of glutamine synthetase in the cyanobionts of the lichens *Peltigera aphthosa* and *Peltigera canina*, *Lichen Physiol. Biochem.*, 1, 27, 1986.

175a. **Rai, A. N., Borthakur, M., Singh, S., and Bergman, B.**, *Anthoceros-Nostoc* symbiosis: immunoelectronmicroscopic localization of nitrogenase, glutamine synthetase, phycoerythrin and ribulose 1,5-biphosphate carboxylase/oxygenase in the cyanobiont and the cultured (free-living) isolate Nostoc 7801, *J. Gen. Microbiol.*, 135, 385, 1989.

176. **Nierzwicki-Bauer, S. A. and Haselkorn, R.**, Differences in mRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*, *EMBO J.*, 5, 29, 1986.

177. **Bernard, T.**, Contribution à l'étude du metabolisme azote des lichens. Activité de la glutamate-decarboxylase de cinq espèces de la famille des Stictacées, *C.R. Acad. Sci. Paris Ser. D.*, 269, 823, 1969.

178. **Bernard, T. and Goas, G.**, Contribution à l'étude du metabolisme des lichens. Mise en evidence de quelques transaminases; activité de la glutamate oxaloacetate transaminase dans cinq espèces de la famille des Stictacées, *C.R. Acad. Sci. Paris Ser. D.*, 269, 1657, 1969.

179. **Stewart, W. D. P.**, Some aspects of structure and function in N_2-fixing cyanobacteria, *Annu. Rev. Microbiol.*, 34, 497, 1980.

180. **Bergman, B., Lindblad, P., and Rai, A. N.**, Nitrogenase in free-living and symbiotic cyanobacteria: immunoelectron microscopic localization, *FEMS Microbiol. Lett.*, 35, 75, 1986.

181. **Millbank, J. W.**, Aspects of nitrogen metabolism in lichens, in *Lichenology: Progress and Problems*, Brown, D. H., Hawksworth, D. L., and Bailey, R. H., Eds., Academic Press, London, 1976, 441.

182. **Rogers, R. W., Lange, R. T., and Nicholas, D. J. D.**, Nitrogen fixation by lichens of arid soil crusts, *Nature (London)*, 209, 96, 1966.

183. **Bond, G. and Scott, G. D.**, An examination of some symbiotic systems for fixation of nitrogen, *Ann. Bot.*, 19, 67, 1955.

184. **Fogg, G. E. and Stewart, W. D. P.**, In situ determination of biological nitrogen fixation in Antartica, *Br. Antarct. Surv. Bull.*, 15, 39, 1968.

185. **Sheridan, R. P.**, Impact of emmissions from coal fired electricity generating facilities on N_2-fixing lichens, *Bryologist*, 82, 54, 1979.

186. **Henriksson, E. and Simu, B.**, Nitrogen fixation by lichens, *Oikos*, 22, 119, 1971.

187. **Kelly, B. B. and Becker, V. E.**, Effects of light intensity and temperature on nitrogen fixation by *Lobaria pulmonaria, Sticta weigelii, Leptogium cyanescens*, and *Collema subfurvum*, *Bryologist*, 78, 350, 1975.

188. **Stewart, W. D. P.**, Algal fixation of atmospheric nitrogen, *Plant Soil*, 32, 555, 1970.

189. **Denison, W. C.**, *Lobaria oregana*, a nitrogen fixing lichen in old growth Douglas fir forests, in *Symbiotic Nitrogen Fixation in the Management of Temperate Forests*, Gordon, J. C., Wheeler, C. T., and Perry, D. A., Eds., Oregon State University Press, Corvallis, 1979, 266.

190. **Millbank, J. W. and Kershaw, K. A.**, Nitrogen metabolism in lichens. III. Nitrogen fixation by internal cephalodia of *Lobaria pulmonaria*, *New Phytol.*, 69, 595, 1970.

191. **Kershaw, K. A.**, Dependence of the level of nitrogenase capacity on the water content of the thallus in *Peltigera canina, Peltigera evansina, Peltigera polydactyla* and *Peltigera praetextata*, *Can. J. Bot.*, 52, 1423, 1974.

192. **Millbank, J. W.**, The assessment of nitrogen fixation and throughput by lichens. I. The use of a controlled environmental chamber to relate acetylene reduction estimates to nitrogen fixation, *New Phytol.*, 89, 647, 1981.

193. **MacFarlane, J. D. and Kershaw, K. A.**, Physiological-environmental interactions in lichens. IX. Thermal stress and lichen ecology, *New Phytol.*, 84, 669, 1980.

194. **Kallio, P., Suhonen, S., and Kallio, H.**, The ecology of nitrogen fixation in *Nephroma arcticum* and *Solorina crocea*, *Rep. Kevo Subarct. Res. Stn.*, 9, 7, 1972.

195. **Kallio, S.**, The ecology of nitrogen fixation in *Stereocaulon paschale*, *Rep. Kevo Subarct. Res. Stn.*, 10, 34, 1973.

196. **Millbank, J. W.**, Nitrogen fixation by lichens, in *Current Developments in Biological Nitrogen Fixation*, Subba Rao, N. S., Ed., Oxford and IBH Publ. Co., New Delhi, 1984, 197.

197. **Hardy, R. W. F., Burns, R. C., and Holsten, R. D.**, Application of the acetylene-ethylene assay for measurement of nitrogen fixation, *Soil Biol. Biochem.*, 5, 47, 1973.

198. **Rennie, R. J., Rennie, D. A., and Fried, M.**, Concepts of ^{15}N usage in dinitrogen fixation studies, in *Isotopes in Biological Dinitrogen Fixation*, International Atomic Energy Agency, Vienna, 1978, 107.

199. **Bergersen, F. J.**, The quantitative relationship between nitrogen fixation and the acetylene reduction assay, *Aust. J. Biol. Sci.*, 23, 1015, 1970.

200. **Schubert, K. R. and Evans, H. J.,** Hydrogen evolution: a major factor affecting nitrogen fixation in nodulated symbionts, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1207, 1976.
201. **Graham, B. M., Hamilton, R. D., and Campbell, N. E. R.,** Comparison of the nitrogen-15 uptake and acetylene reduction methods for estimating the rates of nitrogen fixation by fresh water blue-green algae, *Can. J. Fish. Aquat. Sci.*, 37, 488, 1980.
202. **Bothe, H., Neuer, G., Kalbe, I., and Eisbrenner, G.,** Electron donation and hydrogenase in nitrogen-fixing microorganisms, in *Nitrogen Fixation*, Stewart, W. D. P. and Gallon, J. R., Eds., Academic Press, London, 1980, 83.
203. **Horstmann, J. L., Denison, W. C., and Sivester, W. B.,** $^{15}N_2$-fixation and molybdenum enhancement of acetylene reduction by *Lobaria* spp., *New Phytol.*, 92, 235, 1982.
204. **MacFarlane, J. D., Maikawa, E., Kershaw, K. A., and Oaks, A.,** Environmental-physiological interactions in lichens. I. The interaction of light/dark periods and nitrogenase activity in *Peltigera polydactyla, New Phytol.*, 77, 705, 1976.
205. **Stewart, W. D. P.,** Blue-green algae, in *The Biology of Nitrogen Fixation*, Quispel, A., Ed., North-Holland, Amsterdam, 1974, 202.
206. **Rai, A. N., Rowell, P., and Stewart, W. D. P.,** Mycobiant-cyanobiont interactions during dark nitrogen fixation by the lichen *Peltigera aphthosa, Physiol. Plant.*, 57, 285, 1983.
207. **Singh, H. N., Rai, U. N., Rao, V. V., and Bagchi, S. N.,** Evidence for ammonia as an inhibitor of heterocyst and nitrogenase formation in the cyanobacterium *Anabaena cycadeae, Biochem. Biophys. Res. Commun.*, 111, 180, 1983.
208. **Mackerras, A. H. and Smith, G. D.,** Evidence for direct repression of nitrogenase by ammonia in the cyanobacterium *Anabaena cylindrica, Biochem. Biophys. Res. Commun.*, 134, 835, 1986.
209. **Ownby, J. D.,** Effects of amino acids on methionine-sulphoximine-induced heterocyst formation in *Anabaena, Planta*, 136, 277, 1977.
210. **Ladha, J. K., Rowell, P., and Stewart, W. D. P.,** Effects of 5-hydroxylysine on acetylene reduction and NH_4^+-assimilation in the cyanobacterium *Anabaena cylindrica, Biochem. Biophys. Res. Commun.*, 83, 688, 1978.
211. **Akkermans, A. D. L., Huss-Danell, K., and Roelofsen, W.,** Enzymes of the tricarboxylic acid cycle and the malate aspartate shuttle in the N_2-fixing endophyte of *Alnus glutinosa, Physiol. Plant.*, 53, 289, 1981.
212. **Henriksson, E.,** Nitrogen fixation by a bacteria-free symbiotic *Nostoc* strain from *Collema, Physiol. Plant.*, 4, 542, 1951.
213. **Millbank, J. W.,** Nitrogen metabolism in lichens. V. The forms of nitrogen released by the blue-green phycobiont in *Peltigera* spp., *New Phytol.*, 73, 1171, 1974.
214. **Kershaw, K. A. and Millbank, J. W.,** Nitrogen metabolism in lichens. II. The partitioning of cephalodial-fixed nitrogen between the mycobiant and the phycobiont of *Peltigera aphthosa, New Phytol.*, 69, 75, 1970.
215. **Rai, A. N., Rowell, P., and Stewart, W. D. P.,** Glutamate synthase activity of heterocysts and vegetative cells of the cyanobacterium *Anabaena variabilis* Kütz, *J. Gen. Microbiol.*, 128, 2203, 1982.
216. **Thomas, J., Meeks, J. C., Wolk, C. P., Shaffer, P. W., Austin, S. M., and Chien, W. S.,** Formation of glutamine from [^{13}N] ammonia, [^{13}N] dinitrogen and [^{14}C] glutamate by heterocysts isolated from *Anabaena cylindrica, J. Bacteriol.*, 129, 1545, 1977.
217. **Peters, G. A., Mayne, B. C., Ray, T. B., and Toia, R. E., Jr.,** Physiology and biochemistry of the *Azolla-Anabaena* symbiosis, in *Nitrogen and Rice*, International Rice Research Institute, Las Banos, Philippines, 1979, 325.
218. **Weiss, M. G. and Luria, S. E.,** Reduction of membrane potential, an immediate effect of colicin K, *Proc. Natl. Acad. Sci. U.S.A.*, 75, 2483, 1978.
219. **Reed, R. H., Rowell, P., and Stewart, W. D. P.,** Characterization of the transport of potassium ions in the cyanobacterium *Anabaena variabilis* Kütz, *Eur. J. Biochem.*, 116, 323, 1981.
220. **Reed, R. H., Rowell, P., and Stewart, W. D. P.,** Uptake of potassium and rubidium ions by the cyanobacterium *Anabaena variabilis, FEMS Microbiol. Lett.*, 11, 223, 1981.
221. **Hawkesford, M. J., Reed, R. H., Rowell, P., and Stewart, W. D. P.,** Nitrogenase activity and membrane electrogenesis in the cyanobacterium *Anabaena variabilis* Kütz, *Eur. J. Biochem.*, 115, 519, 1981.
222. **Hawkesford, M. J., Reed, R. H., Rowell, P., and Stewart, W. D. P.,** Nitrogenase activity and membrane electrogenesis in the cyanobacterium *Plectonema boryanum, Eur. J. Biochem.*, 127, 63, 1982.
223. **Musgrave, S. C., Kerby, N. W., Codd, G. A., and Stewart, W. D. P.,** Sustained ammonia production by immobilized filaments of the nitrogen-fixing cyanobacterium *Anabaena* 27893, *Biotechnol. Lett.*, 4, 647, 1982.
224. **Stewart, W. D. P., Codd, G. A., and Rai, A. N.,** H_2 production from sunlight, air and water by N_2-fixing systems involving cyanobacteria, in *Photochemical, Photoelectrochemical and Photobiological Processes*, Vol. 2, Hall, D. O., Palz, W., and Pirrwitz, D., Eds., D. Reidel, Dordrecht, Netherlands, 1983, 214.

225. **Gallon, J. R. and Chaplin, A. E.,** *An Introduction to Nitrogen Fixation,* Cassell Educational, London, 1987, 14.
226. **Murray, P. A. and Zinder, S. H.,** Nitrogen fixation by a methanogenic archaebacterium, *Nature (London),* 312, 284, 1984.
227. **Belay, N., Sparling, R., and Daniels, L.,** Dinitrogen fixation by a thermophilic methanogenic bacterium, *Nature (London),* 312, 286, 1984.
228. **Yamada, T. and Sakaguchi, K.,** Nitrogen fixation associated with a hot spring green alga, *Arch. Microbiol.,* 124, 161, 1980.
229. **Weathers, P. J., Danielli, J. F., Bradley, P. M., Hebb, P. M., Miller, J. E., and Pesano, R. L.,** Possible evidence for novel metabolism of nitrogen gas by a green alga, *Physiol. Plant.,* 61, 441, 1984.
230. **Rai, A. N. and Kumar, H. D.,** Nitrogen-fixing cyanobacteria in the phyllosphere, *SGM Quart.,* 7, 106, 1980.
231. **Davey, M. R. and Power, J. B.,** Polyethylene glycol-induced uptake of microorganisms into higher plant protoplasts: an ultrastructural study, *Plant Sci. Lett.,* 5, 269, 1975.
232. **Burgoon, A. C. and Bottino, P. J.,** Uptake of nitrogen-fixing blue-green alga *Gloeocapsa* into protoplasts of tobacco and maize, *J. Hered.,* 67, 223, 1976.
233. **Meeks, J. C., Malmberg, R. L., and Wolk, C. P.,** Uptake of auxotrophic cells of a heterocyst-forming cyanobacterium by tobacco protoplasts, and the fate of their association, *Planta,* 139, 55, 1978.
234. **Ahmadjian, V., Russell, L. A., and Hildreth, K. C.,** Artificial reestablishment of lichens. I. Morphological interactions between the phycobiont of different lichens and the mycobionts of *Cladonia cristatella* and *Lecanora crysoleuca, Mycologia,* 72, 73, 1980.
235. **Ahmadjian, V. and Jacobs, J. B.,** Relationship between fungus and alga in the lichen *Cladonia cristatella* Tuck., *Nature (London),* 289, 169, 1981.
236. **Culberson, C. F. and Ahmadjian, V.,** Artificial reestablishment of lichens. II. Secondary products of resynthesized *Cladonia cristatella* and *Lecanora crysoleuca, Mycologia,* 72, 90, 1980.

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Chapter 3

CYANOBACTERIAL-BRYOPHYTE ASSOCIATIONS

John C. Meeks

INTRODUCTION

The division Bryophyta includes three classes of small terrestrial plants: Musci (mosses), Hepaticae (liverworts), and Anthocerotae (hornworts). Members of the division are characterized, among other properties, by attachment of the sporophyte to the gametophyte (Figure 1A), the lack of lignified tissue, presence of rhizoids rather than true roots, and a gametophyte growth pattern that is generally close to the substratum.[1] The substratum may be rock, the surface of vascular plants, or soil, with growth occurring under conditions of high moisture. Analysis of the nucleotide sequence of 5S ribosomal RNA indicates a high degree of evolutionary relatedness within the division, but also that the liverworts and mosses have evolved in different directions from the hornworts.[2] In natural habitats, eukaryotic algae and cyanobacteria typically grow in association with bryophytes.[3]

Bryophyte associations with nitrogen-fixing cyanobacteria are of specific interest because the cyanobacteria function in supplying fixed nitrogen to the plant tissue and are, in many ways, analogous to the symbiotic relationship between agronomically important legumes and rhizobia. The proof of such a physiological function was presented in 1955 by Bond and Scott[4] when they demonstrated active nitrogen fixation in the liverwort *Blasia pusilla-Nostoc* sp. association using $^{15}N_2$ and implied transfer of fixed nitrogen from *Nostoc* to the plant tissue. A consequence of this functional relationship is the general categorizing of bryophyte-cyanobacteria associations as symbiotic. Reference to symbiotic association will be made throughout this review. However, following the lead of Bond and Scott, the useage is qualified such that herein "the term symbiosis does not necessarily imply anything more than association of organisms".[4] Rodgers[5] was unable to identify a physiological advantage to a *Nostoc* sp. when in association with *B. pusilla* compared to free-living culture. Sprent and Raven[6] suggested that the liverwort and hornwort associations with cyanobacteria are the most ancient, yet organizationally primitive, of all nitrogen-fixing symbioses; they may not have evolved the mutualistic metabolic interactions seen in associations involving heterotrophic diazotrophs.

In general, the cyanobacteria form epiphytic associations with mosses and are endophytic with liverworts and hornworts, but there are exceptions (Table 1). The associations appear to be confined to the bryophyte gametophore in all cases and primarily involve species of *Nostoc*. Epiphytically associated cyanobacteria tend to be morphologically similar to growth forms in free soil, while those in specific endophytic association have distorted vegetative cell shapes and higher frequencies of heterocysts.

The organizational approach in this review is to discuss the current information on the predominantly epiphytic cyanobacteria-moss associations and then to combine a more detailed discussion of the distinctly endophytic *Nostoc* associations with liverworts and hornworts. The literature accounts date primarily from 1955 onward; references to earlier descriptive reports of bryophyte associations were given by Millbank[7] and Stewart et al.[8]

MOSSES

All studies on the association of nitrogen-fixing cyanobacteria with Musci have been done in the natural habitat or on samples collected from those habitats. For purposes of discussion, the studies can be categorized with respect to two general habitats: (1) mire or bog and (2) grassland or slash-burn forest soils.

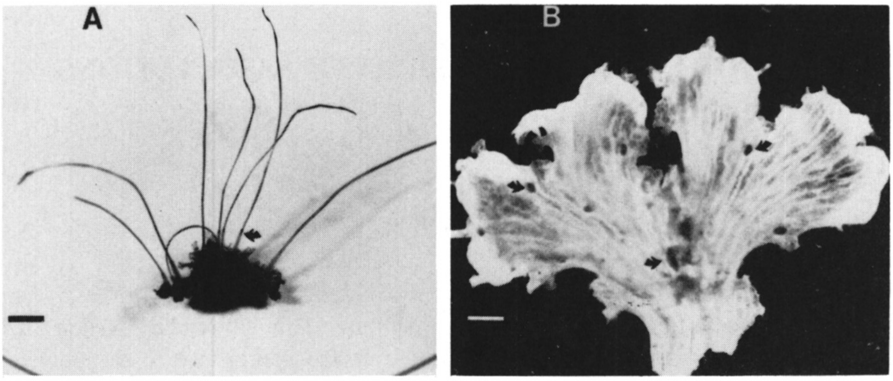

FIGURE 1. Photomicrographs of *Anthoceros punctatus-Nostoc* sp. symbiotic association. (A) Sporophytes attached to gametophytes of *A. punctatus* samples collected from the natural habitat. The gametophytes generally occur in clusters or rosettes and each gametophyte may produce one to three sporophytes. The arrow points to the distinct thallus calyptra at the base of the sporophytes. Marker represents 500 mm. (B) *A. punctatus-Nostoc* sp. strain 7801 tissue reconstituted in pure culture and grown submerged in liquid medium with N_2 as the nitrogen source and photographed with darkfield microscopy. Arrows point to selected colonies of symbiotic *Nostoc* sp. strain 7801. Marker represents 150 mm.

TABLE 1
Cyanobacterial-Bryophyte Associations

Bryophyte	Type of association	Ref.
Mosses		
Sphagnum riparium	Epiphytic and endophytic	9,10,11,12
S. linderbergii	Epiphytic and endophytic	9,10,11,12
Drepanocladus exannulatus	Epiphytic	10,12
D. trichophyllus	Epiphytic	11
Polytrichum commune	Epiphytic	13
Ceratodon purpureus	Epiphytic	14,15
Funaria hygrometrica	Epiphytic	14,16
Bryum argenteum	Epiphytic	17
Weisia controversa	Epiphytic	17
Liverworts		
Blasia pusilla	Endophytic	4,18,19,20
Cavicularia densa	Endophytic	18
Marchantia berteroana	Epiphytic?	14
Porella navicularis	Epiphytic	21
Hornworts		
Anthoceros laevis	Endophytic	22
A. husnotii	Endophytic	22
A. punctatus	Endophytic	19,20,23
Phaeoceros laevis	Endophytic	19
Notothylas sp.	Endophytic	24
Dendroceros sp.	Endophytic	24

STUDIES IN MIRE HABITATS

Mires or bogs are often dominated by species of *Sphagnum* (Sphagnidae) and *Drepanocladus* (Bryidae), especially in subarctic regions.[12] Except for two species of *Sphagnum*, the cyanobacteria form epiphytic associations with mosses (Table 1). Stewart[9] first described the endophytic association of a *Sphagnum* sp. with a nitrogen-fixing cyanobacterium, identified as *Hapalosiphon* sp., and reported fixation of $^{15}N_2$ by the association. The cyanobacteria were localized in hyaline cells of the moss. By definition, hyaline cells are clear or translucent and appear to be dead; their function, if any, is unknown.[1] The intracellular localization of a nitrogen-fixing cyanobacterium, identified as *Nostoc* sp., was confirmed by Granhall and Selander[10] and Granhall and van Hofsten[11] and shown to be confined to *Sphagnum riparium* and *S. lindbergii*. The *Nostioc* sp. was morphologically similar to epiphytically associated populations.[11] Because cyanobacteria grow poorly, if at all, at low pH (<4.0 to 5.0),[25] the advantage to *Nostoc* of localization in hyaline cells was suggested to be buffering of the general acidity in mire habitats by plant metabolites.[11] The mode of entry of *Nostoc* into the hyaline cells is unknown.

Endophytic *Nostoc* associations with *Sphagnum* are neither as frequent in occurrence nor as active in nitrogen fixation as epiphytic associations.[26] Endophytic associations seem to be confined to the few plants that grow out of the bog water, while the majority, submerged plants, have primarily epiphytic *Nostoc*.[26] The annual rates of nitrogen fixation in subarctic mires were estimated to be 0.2, 0.2, and 9.4 g N per square meter per year for free-living *Nostoc, Sphagnum* with intracellular *Nostoc,* and *Sphagnum* with epiphytic *Nostoc,* respectively.[27] *Drapanocladus trichophyllus*[10] and *D. exannulatus,*[12] both with epiphytic *Nostoc,* showed rates of nitrogen fixation (acetylene reduction) comparable to *Sphagnum* species with epiphytic *Nostoc.*

Collectively, the *Sphagnum* and *Drapanocladus* studies[10-12,26,27] indicate substantial input of fixed nitrogen from epiphytically associated *Nostoc* sp. in those limited mire habitats. Granhall and Lindberg[28] subsequently showed high rates of cyanobacterial acetylene reduction in *Sphagnum* communities on the floor of a mixed pine and spruce forest. Although the extrapolated rates of fixation were not as high as the mire sites, the study implied that epiphytic nitrogen-fixing cyanobacteria-*Sphagnum* associations might develop in a variety of environments.

Using $^{15}N_2$, Basilier[26] demonstrated transfer of nitrogen fixed by epiphytic *Nostoc* to the *Sphagnum* growing apex, thus providing proof of a metabolic interaction. Basilier[26] also normalized rates of acetylene reduction to heterocyst counts and calculated that epiphytically associated *Nostoc* were 14 to 22 times more active than free-living cultures. Although the culture conditions were not defined, those results at least imply a *Sphagnum*-dependent alteration in *Nostoc* metabolism. The extent and mechanism(s) of the metabolic alteration in associated *Nostoc* have not been studied.

STUDIES IN GRASSLANDS AND SLASH-BURN SITES

Vlassak et al.[15] were the first to show acetylene reduction by cyanobacteria in epiphytic association with the cosmopolitan moss *Ceratodon purpureus* (Bryidae). The habitat was a natural prairie system in Canada. Although several cyanobacteria were variously present on *C. purpureus,* only *Nostoc* sp. was common to all samples. The rates of acetylene reduction associated with *C. purpureus* were estimated to be 500-fold lower than those of cyanobacterial mats (crusts) in similar grasslands;[15] by extrapolation, the associated rates were also about 100- to 150-fold lower than those recorded with *Sphagnum* in mires. Acetylene reduction associated with *C. purpureus* was confirmed by Brasell et al.,[14] in this case in forest soils of Tasmania after clear cutting of the trees and burning of the resulting slash. The acetylene reduction rates associated with *C. purpureus* in this habitat reached a maximum more than 2 years after the burn and were five- to sevenfold lower than the maximal rates of *Sphagnum*-associated mire activities.

Brasell et al.[14] also detected acetylene reduction associated with *Funaria hygrometrica* (Bryidae) in the slash-burn sites. The maximal rates of fixation were about 1.5-fold lower than of *C. purpureus*. However, *F. hygrometrica* showed the maximal rates within 1.5 years following the burn. The differences between the two mosses in the time after the burn to express maximal nitrogen fixation was assumed to reflect the rate of colonization and succession. By use of autofluorescence and epifluorescence microscopy, Scheirer and Brasell[16] established the epiphytic localization of cyanobacteria on *F. hygrometrica* and tentatively identified *Nostoc* sp. and/or *Anabaena* sp. as the prevalent epiphytes.

Nostoc and *Anabaena* were also observed to be present as epiphytes on *Bryum argenteum* (Bryidae) and *Weisia controversa* (Bryidae) collected in fescue grass plots.[17] In this study the rates of acetylene reduction in the cyanobacteria-associated mosses were reported to be two- to threefold greater than that of cyanobacteria alone. However, because the rates were not normalized to a standard parameter, it is not possible to compare the results to other studies.

SUMMARY

The above reports notwithstanding, the documented instances of *Anabaena* or *Nostoc* association with mosses remain rare, especially with respect to the variety of moss habitats and taxonomic groups. It is possible that more extensive and rigorous surveys will identify additional associations. Studies to examine the cyanobacterial specificity of the known moss associations, possible physiological adaptation, and metabolite exchange are clearly needed to understand their environmental impact and evolutionary implications.

LIVERWORTS AND HORNWORTS

The liverworts are taxonomically diverse, with at least 330 defined genera.[1] However, like the mosses, associations with nitrogen-fixing cyanobacteria are exceedingly rare; there are two documented instances each of Hepaticae genera with endophytic and epiphytic associations (Table 1). The hornworts are represented by six extant genera.[1] Although textbooks tend to imply that the Anthocerotae invariably have endophytic nitrogen-fixing cyanobacteria in association, there are no published reports of such an association with *Megaceros* spp. or *Folioceros* spp. In all cases of endophytic association, the cyanobacteria are considered to be species or strains of *Nostoc*. The *Nostoc* communities are macroscopically visible as dark round spots (0.4 to 0.7 mm in diameter) in the gametophyte tissue (Figure 1B) and have the appearance of a distinctly organized structure, thus the general reference to symbiotic *Nostoc* colonies.[20,23]

Morphologically and physiologically, the endophytic associations of *Nostoc* with liverworts and hornworts appear to be similar. Therefore, these associations are discussed without specific regard to taxonomic class. The more recently described epiphytic liverwort associations are considered first.

EPIPHYTIC ASSOCIATIONS

Dalton and Chatfield[21] used light microscopy to detect the presence of epiphytic *Nostoc* on the leaves of the leafy liverwort *Porella*. The colonization was especially prevalent on *Porella navicularis*, a species endemic to western North America. The associated *Nostoc* was active in acetylene reduction, but with a vegetative cell morphology and heterocyst frequency similar to a free-living population. Out of 68 *P. navicularis* samples collected from various sites, 85% were positive for acetylene reduction. The highest rates observed were similar to those recorded for the *Nostoc* association with *Ceratodon purpureus* in slash burn soils.[14]

The maximal rate of acetylene reduction associated with *Marchantia berteroana* in the slash burn soil[14] was about one third that observed with *P. navicularis* or *C. purpureus*. Unfortunately, neither identification nor localization of the nitrogen-fixing organism in association with *M. berteroana* was attempted, but because of the lack of correlation between acetylene reduction and soluble organic matter, it was assumed to be cyanobacterial.[14]

As with moss associations, physiological and taxonomic details of the epiphytic association of cyanobacteria with liverworts are lacking. However, based on the information available, it would appear that epiphytic associations with bryophytes in general occur more often than previously thought.

ENDOPHYTIC ASSOCIATIONS

The endophytic associations of *Nostoc* spp. with liverworts and hornworts have been most highly studied because: (1) they are easily adaptated to laboratory culture, (2) the associations can be reconstituted from the separately cultured partners, (3) there is interest in the cause of the pronounced morphological changes in the *Nostoc,* and (4) the *Nostoc* is distinctly localized within "slime cavities" in the gametophyte thallus, which facilitates tissue selection in structural and ultrastructural studies. The formation of a functional N_2-fixing endophytic association probably results from a continuum of interactions between the two partners of the symbiosis. Nevertheless, work over the past 7 years in our laboratory on the hornwort *Anthoceros punctatus-Nostoc* spp. association has indicated that the interactions between the partners can be categorized into two major stages, each with two distinct substages, that are amenable to experimentation: (1) initial establishment of the association, i.e., (i) formation of *Nostoc* infective units and (ii) infection; and (2) regulation of (i) growth and metabolism and (ii) differentiation of associated *Nostoc.* The following discussion of the endophytic associations is organized around those experimental stages and preceded by a summary of culture conditions.

BRYOPHYTE CULTURE

Cultures of gametophyte tissues of liverworts and hornworts, collected in the field and containing associated cyanobacteria, can be maintained in the laboratory.[20] Such cultures reportedly grew best on solid substratum (agar or Peralite) at low light and at 10 to 15°C; they did not grow when submerged.[20]

Axenic cultures of bryophytes are readily obtained by surface sterilization of spores contained within unopened sporophytes (Figure 1A)[23,29,30] or of fragments of gametophyte tissue.[19,30] Surface-sterilized spores germinate and grow on a variety of defined salts media, solidified with 1.0 to 1.5% agar.[23,29-31]

Certain liverworts, e.g., *Sphaerocarpos donnellii*[32] and the hornwort *Anthoceros punctatus*[23] can be adapted to pure culture in submerged liquid medium. Submerged growth of *A. punctatus* was dependent upon adequate buffering of the medium, agitation at approximately 100 rpm on a platform shaker, and moderate temperature (20°C).[23] Doubling times of 5 d for *A. punctatus* with NH_4^+ as the nitrogen source, or 10 d for *A. punctatus-Nostoc* sp. strain 7801 with N_2 only, can be obtained at light intensities of 7 W/m^2 in liquid medium supplemented with 0.5% (w/v) glucose or under 340 W/m^2 with 5% (v/v) CO_2 as the sole carbon source.[23,33] In addition to a relatively rapid growth rate, submerged liquid culture allows for experimental flexibility in supplementation with growth factors or inhibitors and in reconstitution of the symbiotic association from separately cultured partners.

ESTABLISHMENT OF THE SYMBIOTIC ASSOCIATION

Formation of *Nostoc* Infective Units

Stewart et al.[8] and Enderlin and Meeks[23] commented on the apparent correlation between symbiotically competent cyanobacteria and the presence of hormogonia. Hormogonia are defined as filaments that lack heterocysts and have one or more of the following properties: rapid gliding motility, small cell size, different cell shape, and gas vacuolation.[34] Hormogonia are produced at a low frequency as part of the life cycle of certain Nostocales and are what superficially distinguishes *Nostoc* from *Anabaena.*[34] They arise by a stimulation of cell division, uncoupled from a comparable increase in biomass, with an accompanying detachment of existing heterocysts. The hormogonia of symbiotic *Nostoc* strains are highly motile, small-celled filaments that lack gas vacuoles; they are shown compared to vegetative filaments in Figure 2.

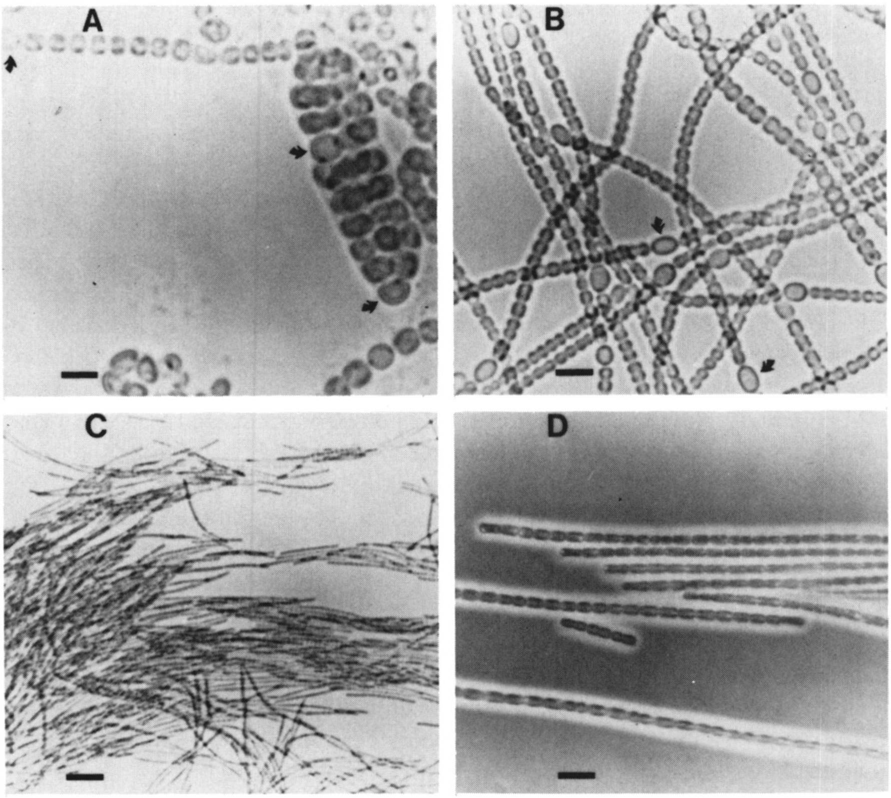

FIGURE 2. Photomicrographs of free-living *Nostoc* sp. strain 7801. (A) Strain 7801 in pure culture photographed in 1979, 1 y after isolation from *Anthoceros puncatus* tissue. Arrows point to heterocysts. Marker represents 5μm. (B) Strain 7801 8 y after isolation and continued subculture in the free-living state in minimal salts medium. Note the absence of enlarged cells and coiled filaments within sheath material in the samples in panel B that were characteristic of the culture in panel A. Arrows point to heterocysts. Marker represents 5μm. (C) and (D) Hormogonia filaments formed 24 h after exposure of *Nostoc* sp. strain 7801 to *A. punctatus*. Panel C illustrates the extent of hormogonia formation, while the filaments in panel D are at higher magnification. Markers represent 20μm in panel C and 5μm in panel D. Panels A, B, and C are bright field microscopy and D is phase contrast.

Immediate coculture of several *Nostoc* strains with *A. punctatus* in the absence of combined nitrogen results in massive conversion of the *Nostoc* vegetative filaments into hormogonia.[35] The stimulation of hormogonia formation was dependent upon an extracellular, low molecular weight, heat-labile product of *A. punctatus* metabolism. Production of the extracellular product was inhibited by culture of *A. punctatus* in excess NH_4^+. Induced *Nostoc* sp. strain 7801 remained in the hormogonia state for less than 48 h before it differentiated new heterocysts and initiated growth. Once induced into the hormogonia state, the *Nostoc* require a growth period of at least 2 weeks before greater than 90% of the filaments again form hormogonia in the presence of *A. punctatus* or its products.

The specific activities of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and glutamine synthetase (GS), the primary enzymes of inorganic carbon and nitrogen assimilation, respectively, did not change significantly during hormogonia formation. *In vivo,* hormogonia assimilated CO_2 and exogenous NH_4^+ at about 65% of the rate of vegetative filaments.[35] Thus, other than detachment of heterocysts and concomitant loss of nitrogen fixation, these hormogonia appear physiologically similar to vegetative filaments and may not be metabolically inert, strictly growth-precursor cells as previously suggested.[36]

All symbiotically competent *Nostoc* strains tested during coculture with *A. punctatus* convert 50 to 99% of their vegetative filaments into hormogonia within 24 h of exposure. Gliding motility coupled with chemotactic response may be the most important characteristics of hormogonia of symbiotically competent *Nostoc* strains in locating the "slime pores", or cavity openings, in the gametophyte thallus and colonizing the cavities. Although gliding motility has been demonstrated, there is no current evidence of chemotactic behavior in the *Nostoc* spp. and *A. punctatus* interaction. Moreover, hormogonia formation and gliding motility do not unequivocally identify a symbiotically competent *Nostoc* strain. *Nostoc* sp. strain ATCC 27896 forms extensive hormogonia in the presence of *A. punctatus*,[35] but it does not develop a symbiotic association within the operational time frame (see below).[23]

Infection of Gametophyte Tissue by *Nostoc* spp.

The endophytic associations of *Nostoc* spp. with liverworts and hornworts have been reconstituted in the laboratory from the separately cultured partners in three published instances.[19,20,23] Ridgway reported experimental reconstitution using the hornworts *A. husnoti*, *A. punctatus*, and *Phaeoceros laevis* as host plants.[19] This pioneering study not only provided evidence of laboratory reconstitution of a bryophyte association, but also indicated the broad specificity of cyanobacteria in such associations and suggested that they may exclusively involve species of *Nostoc*. As determined by macroscopic observation, the hornwort associations were reconstituted during prolonged (125 d) coculture on agar-solidified combined nitrogen-free medium with a wide variety of *Nostoc* isolates. Symbiotically competent *Nostoc* strains included isolates from *P. laevis* and *Blasia pusilla*, as well as ones from associations with cycads (*Cycas circinalis*, *Dion* sp., *Encephalartos altensteinii*, and *Stangeria paradoxa*) and the angiosperm *Gunnera chilensis*, plus two strains of unknown origin; one isolate from the cycad *Dion* sp. failed to form an association with the hornworts. The competent strains associated with the three hosts equally well, except for one strain identified as *Nostoc muscorum* that infected *P. laevis* but did not associate with either species of *Anthoceros*. Two cultures listed as *Anabaena variabilis* and *A. cylindrica* and a second culture of *N. muscorum* were nonsymbiotic with the hornworts.

Rodgers and Stewart[20] verified the results of Ridgway in reconstitution of *Anthoceros punctatus* with *Nostoc* and also succeeded in establishing association of the liverwort *B. pusilla* with *Nostoc* during coculture on sand substratum. In experiments with *A. punctatus*, *Nostoc* infection of the gametophyte cavity was noted within 72 h of coculture. The competent *Nostoc* strains in association with both host plants were direct and reciprocal isolates from *A. punctatus* and *B. pusilla*, plus an isolate from *Gunnera* sp. Four additional *Nostoc* strains, including isolates from the lichen *Collema tenax* and an unnamed cycad, did not establish symbiotic colonies in either bryophyte thallus. A total of 11 other nitrogen-fixing and nonnitrogen-fixing cyanobacteria of various genera were tested and found unable to associate with *B. pusilla*.

We have confirmed and extended the observations of Ridgway[19] and Rodgers and Stewart[20] on reconstitution of the *A. punctatus* association with a variety of *Nostoc* isolates (Table 2).[23] The criteria of reconstitution in this continuing survey are the presence of symbiotic *Nostoc* colonies in, and N_2-dependent growth of, gametophyte tissue. Operationally, reconstitution must occur within 3 weeks of coculture in submerged liquid medium. Details of the culture of the reconstituted *A. punctatus-Nostoc* spp. associations are given elsewhere.[33]

Nostoc strains obtained from virtually all other cyanobacterial symbiotic associations are competent in forming the *A. punctatus* association, as are a number of soil and pond isolates (Table 2).[23] The broad genetic diversity of *Nostoc* strains that establish a functional symbiosis with *A. punctatus* is illustrated in three of the competent American Type Culture Collection (ATCC) strains; strain 29133, isolated from the cycad *Macrozamia* sp., has less

TABLE 2
Cyanobacterial Strains Tested for Reconstitution in the *Anthoceros punctatus* Association during Coculture in Submerged Liquid Medium

Strain	Source	Reconstitution
Nostoc 7801	*A. punctatus* (California; original isolate)	+
Nostoc 8020	*Anthoceros* sp. (New Zealand)	+
Nostoc 8101	*Anthoceros* sp. (Washington State)	+
Nostoc 7901	*Gunnera* sp. (New Zealand)	+
Nostoc 7851	*Peltigera aphthosa* (Oregon)	+
Nostoc 8050	*P. polydactyla* (New Zealand)	+
Nostoc 8071	*Cycas* sp. (New Zealand)	+
Nostoc ATCC 29133	*Macrozamia* sp. (Australia)	+
Nostoc ATCC 27895	Soil isolate (*Anabaenopsis circularis;* Japan)	+
Nostoc ATCC 29150	Pond isolate (California)	+
Nostoc PCC 7812	Free-living isolate (unknown)	+
Nostoc PCC 7906	Free-living isolate (unknown)	+
Nostoc 120	*Azolla caroliniana* (minor symbiont; Ohio/California)	+
Nostoc 123	Soil isolate (Calexico, California)	+
Nostoc Mac	*Macrozamia lucida* (Australia; N_2 fixation minus)	–
Nostoc Mac R1, R2	N_2 fixing revertants of *Nostoc* Mac	–
Nostoc ATCC 27904	Soil isolate (California; N_2 fixation minus)	–
Nostoc ATCC 27904R	N_2 fixing revertant of ATCC 27904	–
Nostoc ATCC 27896	Pond isolate (*Anabaena spiroides;* Israel)	–
Nostoc ATCC 29106	Soil isolate (*Cylindrospermum licheniforme;* England)	–
Nostoc ATCC 29168	Soil isolate (Senegal)	–
Anabaena azollae	*Azolla caroliniana* (Newton/Herman isolate)	–
Anabaena PCC 7120	ATCC 27893 (N. *muscorum;* unknown)	–
A. variabilis	ATCC 29413 (UT 1444; *A. flos-aquae;* unknown)	–
A. cylindrica	ATCC 29414 (UT B381; *A. inequalis;* Utrecht)	–

Note: ATCC = American Type Culture Collection, Rockville, Maryland; PCC = Paris Culture Collection, Institut Pasteur, Paris; UT = Culture Collection of Algae at The Univeristy of Texas, Austin, Texas.

than 35% DNA relatedness with the soil- and pond-isolated strains 27895 and 29150, based on DNA-DNA reassociation.[37] However, a substantial number of strains classified as *Nostoc* by Rippka et al.[34] are nonsymbiotic with *A. punctatus,* even when tested under conditions not requiring a functional N_2-fixing association. The nonsymbiotic strains include ATCC 27896, which has a 65% relatedness with symbiotic strain 29133 according to DNA-DNA reassociation; this was the highest relatedness reported for the *Nostoc* strains examined.[37] An association with *A. punctatus* was not formed by any strain currently classified in the genus *Anabaena* by Rippka et al.,[34] including the culture called *Anabaena azollae* established from *Azolla caroliniana* by Newton and Herman,[38] or by strains that fall into a common *Anabaena-Nostoc* group based on DNA-DNA reassociation.[37] Nevertheless, formation of a functional association with *A. punctatus* was used to selectively isolated symbiotic cyanobacteria from *Azolla caroliniana.*[39] According to the pattern of endonuclease restriction fragments hybridizing to several gene probes, the three independent isolates obtained by forced symbiosis with *A. punctatus* are the same organism, but it is distinct from the major symbiont in *Azolla caroliniana* and other putative symbiotic isolates from *Azolla* species.[39]

Enderlin and Meeks[23] used pigmentation characteristics and spontaneously arising mutants resistant to metabolic analogs to examine the competitive relationships between three *Nostoc* strains infecting *A. punctatus.* Resistance to analogs due to changes in transport (L-methionine-DL-sulfoximine, or MSX) or periplasmic enzymes (5-fluorocytosine, or 5FC) caused an increase in infectiveness of the strain relative to its parental culture. This response was similar to rhizobial mutants with altered infectivity due to changes in outer membranes or cell walls.[40] Whether the similarity in mutant reponse implies a similarity in terms of recognition and infection in the two different symbiotic associations is not known. The results of the competition experiments indicated that *Nostoc* sp. strain 7901 (from *Gunnera* sp.) was clearly more competitive than *Nostoc* sp. strain 7801 (from *A. punctatus*) and slightly more so than *Nostoc* sp. strain ATCC 27895 (free-living isolate). *Nostoc* sp. strain 7801 and *Nostoc* sp. strain ATCC 27895 were equally competitive in infection. This nonlinear relationship suggests that infection is determined by more than one property of the *Nostoc* strains; variations in a single property should result in a linear hierarchy of competitiveness.

Analysis of individual symbiotic *Nostoc* colonies in *A. punctatus* tissue resulting from bi- and tripartite competition experiments indicated that a single *Nostoc* colony can arise from infection by more than one strain.[23] This observation implies that *Nostoc* colonies in gametophyte tissue in the natural habitat could contain multiple strains or different colonies could each contain a different strain, depending upon the numbers and infectiveness of the competent *Nostoc* strains in the immediate environment.

Rodgers and Stewart[20] identified the symbiotically competent *Nostoc* isolates from *A. punctatus* and *B. pusilla* as *Nostoc sphaericum* and the isolate from *Gunnera* sp. as *N. punctiforme*. Based on culture morphology of isolates from *A. punctatus*, *A. husnotii*, and *A. laevis*, Duckett et al.[22] also suggested that the symbiont of these hornworts was *N. sphaericum*, while *N. calcicola* was associated with *B. pusilla*. Watanabe and Kiyohara[18] identified the N_2-fixing cyanobacterium isolated from both *N. pusilla* and *Cavicularia densa* as *N. sphaericum*. The *Nostoc* isolated from *A. punctatus* by Enderlin and Meeks[23] had a morphology consistent with that described for *B. sphaericum*.[41] However, the morphology (but apparently not the physiology) of the isolate in this laboratory has changed during pure culture over the past 10 years (Figure 2A and B). For this reason, as well as the systematic problems enumerated by Rippka et al,[34] the genetic and phenotypic diversity of symbiotically competent *Nostoc* strains, and the nonexclusive, nonlinear competitive relationships between competent strains, we would hesitate to assign a specific epithet based only on morphology to a single *Nostoc* strain isolated from liverwort or hornwort associations.

Collectively, the information from surveys of competent *Nostoc* isolates, alterations in infectiveness by mutation, and nonlinear competitive relationships gives credence to the idea that infection of *A. punctatus* is a function of more than one property of the *Nostoc* strains. Stimulation of *Nostoc* hormogonia formation by *A. punctatus* may serve only to increase the frequency and rate of infection under environmental conditions of nitrogen limitation. The observation that *Nostoc* sp. strain 7801 infects *A. punctatus* during coculture in the presence of NH_4^+, but that the resulting colonies do not increase in size nor is there induction of nitrogenase activity,[23] is also consistent with the suggestion that infection is a property of *Nostoc* and apparently does not require immediate formation of a functional N_2-fixing association.

Morphology of Symbiotically Associated Nostoc

In the liverworts and hornworts, as in other cyanobacterial associations involving a photosynthetic eukaryotic partner, the symbiotic *Nostoc* have a filament and cellular morphology markedly different from free-living cultures.[20,22,23] The symbiotic *Nostoc* vegetative cells enlarge and become spherical. The connections between adjacent vegetative cells and heterocysts and vegetative cells appear to be fragile. Under pressure of a microscopic slide cover slip, freshly isolated *Nostoc* colonies yield predominantly single cells and doublets rather than filaments (Figure 3C).

The most pronounced morphological change in symbiotic *Nostoc* is the increase in heterocyst frequency of N_2-grown associations relative to free-living cultures of the same strain. However, the distorted shapes of vegetative cells make it difficult to distinguish them from heterocysts. Moreover, symbiotic heterocysts tend to have less distinct polar granules

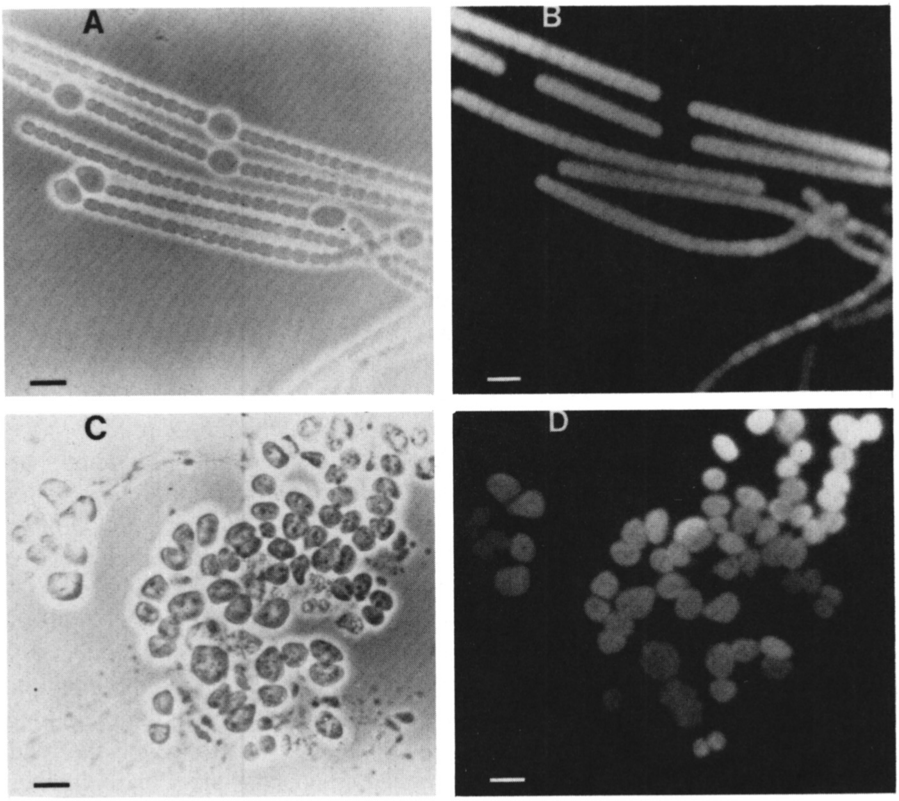

FIGURE 3. Photomicrographs of free-living and symbiotically grown *Nostoc* sp. strain 7801, both with N_2 as the nitrogen source. (A) and (C) utilize phase contrast microscopy of (A) free-living filaments and (C) symbiotic clusters. (B) and (D) are from epifluorescence microscopy; the exciting light of 560 nm was absorbed by phycoerythrin; the emitted light was >600 nm and included fluorescence from phycocyanin, allophycocyanin, and chlorophyll *a*. Note the lack of fluorescence by heterocysts in panel B and the fluorescence from >90% of the cells in panel D; by electron microscopy, about 45% of the symbiotic cells are active and degenerative heterocysts. Marker is 5 μm in all panels.

than free-living cultures and differences in pigmentation between the cell types is muted (Figure 3C and D). Nevertheless, using light microscopy, Rodgers and Stewart[20] and Enderlin and Meeks[23] estimated heterocyst frequencies that ranged from 30% in *B. pusilla*[20] to 43—45% in *A. punctatus*.[20,23] Duckett et al.[22] calculated heterocyst frequencies of 40 to 50% in *Nostoc* associated with *B. pusilla* and three species of *Anthoceros* as based on criteria from electron micrographs. The heterocyst frequency of *Nostoc* sp. strain 7801 in association with *A. punctatus* has been reexamined using ultrastructural criteria to distinguish vegetative cells, heterocysts, and degenerative heterocysts. Based on analysis of about 1200 total cells, 25% appear to be active heterocysts and about 20% have the characteristics of senescent or degenerative heterocysts.[42] Since heterocysts are considered to have a finite lifespan,[43] one would predict the presence of both active and degenerative forms in pure cultures of the symbiotic association, especially with the *Nostoc* confined to a tissue cavity.

Representative ultrastructural characteristics of the cell types are shown in Figure 4. In the submerged liquid cultured *A. punctatus-Nostoc* sp. strain 7801 association, the cyanobacterial vegetative cells contain nitrogen reserves in the form of cyanophycin granules, phycobilisomes, and carboxysomes, as well as numerous glycogen granules. The relative amounts of these cellular inclusions are equal to or greater than those observed in vegetative cells of free-living, N_2-grown cultures. The symbiotic heterocysts lack the listed inclusions,

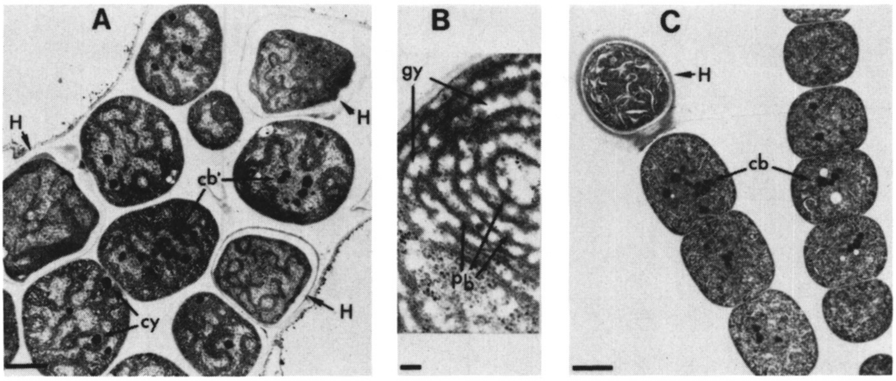

FIGURE 4. Electron micrographs of (A) free-living and (C) symbiotically grown *Nostoc* sp. strain 7801. (B) is a highly magnified section of a vegetative cell of symbiotically grown *Nostoc* sp. strain 7801 showing whorls of thylakoids and the presence of phycobilisomes and glycogen granules. Symbols: H = heterocyst; cb = carboxysome; cy = cyanophycin granule; gy = glycogen granule; pb = phycobilisome. Large spherical electron transparent areas in free-living and symbiotic vegetative cells are sites of polyphosphate granules. Marker is 1.25μm in panels A and C, and 80 nm in panel B.

but may contain phycobiliproteins in a soluble state (Figure 3D). In regard to the presence of phycobilisomes and cyanophycin granules, *Nostoc* sp. strain 7801 associated with *A. punctatus* in pure liquid cultures differs from symbiotic material collected in the field[22] or maintained in the laboratory on solid substratum,[20] in which the *Nostoc* reportedly lack these structures. Cyanophycin and glycogen granules are absent in vegetative cells of cyanobacteria grown under nitrogen and carbon limitation, respectively, and phycobilisomes are rapidly degraded during nitrogen starvation.[44] The presence of these cellular inclusions in the *Nostoc* association with *A. punctatus* under our culture conditions implies that the symbiotic cells are neither nitrogen nor carbon starved.

The bryophytes respond to the endophytic presence of *Nostoc* by an increase in the cavity surface area, in part by proliferation of multicellular filaments of eukaryotic origin.[20,22] These bryophyte filaments have the characteristic morphology of transfer cells and are thought to mediate the interchange of metabolites.[22]

REGULATION OF GROWTH, METABOLISM, AND CELLULAR DIFFERENTIATION OF SYMBIOTICALLY ASSOCIATED *NOSTOC* SPP.

Growth and General Carbon and Nitrogen Metabolism

One would anticipate that in the ancient liverwort and hornwort associations with *Nostoc*, the partners would have evolved adaptations to maintain a degree of balanced growth. Under optimal conditions in the laboratory, symbiont-free *A. punctatus* will double in biomass every 5 d, while reconstituted *A. punctatus-Nostoc* sp. strain 7801 takes 10 d when N_2 is the sole source of nitrogen.[23] In the free-living state and under diazotrophic growth conditions, *Nostoc* sp. strain 7801 will double every 45 h. Thus, to achieve balanced growth of the associated partners, some manner of regulation must be exerted by the bryophyte to slow growth of the *Nostoc*. The nature of the regulatory signals and the specific metabolic systems of *Nostoc* that are targets of the signals are unknown, but these questions are under active investigation. Experience with the *A. punctatus* experimental symbiotic system indicates that some *Nostoc* strains (e.g., *Nostoc* sp. strain 7801) have a stringent response to the unknown signals and establish a relatively stable symbiotic relationship compared to other strains (e.g., *Nostoc* sp. strain ATCC 27895) that tend to grow out of the symbiotic cavities onto the surface of the gametophyte and into the medium. Under the benign liquid culture conditions, all *Nostoc* strains can grow out of the symbiotic cavities; for subsequent physiological experiments, any outgrowth of *Nostoc* is removed by treatment with penicillin.[23,33]

TABLE 3
Summary of Growth and *In Vivo* Carbon and Nitrogen Metabolic Activity of *Nostoc* sp. Strain 7801

	Growth state (N source for growth)		
	Free-living	**Symbiotic**	
Activity	**(N_2)**	**(N_2)**	**(NH_4^+)**
Growth (doubling time)	45 h	<240 h	120—240 h
$^{14}CO_2$ fixation (nmol/min/mg protein)	128.0	15.1	12.6
$^{13}NH_4^+$ assimilation (% total cpm/cpm added/mg protein)	13.9	2.9	1.5
C_2H_2 reduction (nmol/min/mg protein)	6.3	22.4	—

Note: Growth of free-living cultures was calculated from increases in chlorophyll *a*; that of symbiotic cultures was described in the text.

- CO_2 fixation is based on light-dependent, DCMU-sensitive incorporation of $^{14}Co_2$ into cells of free-living or immediately isolated symbiotic colonies;[45] values are means of 9 (free-living), 12 (symbiotic, N_2-grown), and 3 (symbiotic, NH_4^+-grown) replicates.
- NH_4^+ assimilation is based on incorporation of ^{13}N into the sum of glutamine, glutamate, and alanine in methanolic extracts after 5 min of incubation with $^{13}NH_4^+$ by free-living or immediately isolated symbiotic colonies;[46] values are means of 11 (free-living), 8 (symbiotic, N_2-grown) or 3 (symbiotic, NH_4^+-grown) replicates.
- C_2H_2 reduction was determined *in situ*,[23] normalized first to number of *Nostoc* colonies in the gametophyte tissue, then corrected for milligrams protein (0.55 μg) per *Nostoc* colony; for free-living cultures the activity was assayed directly and normalized to protein. Values are means of 80 data points from 13 separate reconstituted *A. punctatus-Nostoc* sp. 7801 samples (symbiotic) or 10 (free-living) replicates.

Because cyanobacteria are distinguished by their oxygenic photosynthetic mechanism for autotrophic growth and the importance of nitrogen metabolism to heterocyst differentiation (as well as to the functional symbiotic relationship), most attention has focused on these processes. The rates of growth and *in vivo* activities of inorganic carbon and nitrogen assimilation by *Nostoc* sp. strain 7801 in free-living culture or in association with *A. punctatus* are summarized in Table 3.

The approximate 240-h doubling time of symbiotic *Nostoc* during N_2-dependent growth was estimated from two indirect observations. One observation was the constant number and size of symbiotic *Nostoc* colonies per unit of growing gametophyte tissue over an extended period of time (>6 y); a constant ratio could only result from rates of *Nostoc* infection and colony development that are similar to gametophyte growth. Second, when *Nostoc* infection was inhibited by penicillin treatment, the size and nitrogen-fixing activity of existing *Nostoc* colonies increased in parallel to gametophyte fresh weight.[23] Because *Nostoc* colonies established in the presence of NH_4^+ tend not to increase in size, it appears that growth of associated *Nostoc* is not as rapid as gametophyte tissue under these culture conditions. However, NH_4^+-treated, N_2-reconstituted associations also tend to break down as a consequence of *Nostoc* outgrowth during long-term culturing.

The *in vivo* activities of photosynthetic CO_2 fixation[45] and exogenous NH_4^+ assimilation[46] of immediately isolated symbiotic *Nostoc* sp. strain 7801 are five- to eightfold lower, approximately in proportion to the decreased growth rate, relative to free-living *Nostoc* sp. strain 7801 (Table 3). We speculate that these *in vivo* assimilatory activities are lower as a consequence, but are not the cause, of the lower growth rate of symbiotic *Nostoc*.

The depressed *in vivo* activities of carbon and nitrogen assimilation by *Nostoc* in association with *A. punctatus* have been verified by measurements *in situ*. In both cases, the *in situ* measurements were made possible by first isolating analog- and herbicide-resistant mutants of *Nostoc* sp. strain 7801 and reconstituting these mutants into the *A. punctatus* association.

Cyanobacteria assimilate NH_4^+, derived from N_2 or NO_3^- or supplied exogenously, almost exclusively by the sequential activities of GS and glutamate synthase (GOGAT);[47] i.e., the GS-GOGAT pathway or glutamate synthase cycle. When in association with *A. punctatus*, *Nostoc* sp. strain 7801 assimilates NH_4^+ by the glutamate synthase cycle,[46] as does *A. punctatus* grown under nitrogen limitation.[48] The glutamate analog, MSX, is an irreversible inhibitor of GS.[49] Assimilation of $[^{13}N]N_2$-derived $^{13}NH_4^+$ into glutamine and glutamate by *A. punctatus* associations reconstituted with wild-type or MSX-resistant *Nostoc* sp. strain 7801 was examined in the absence and presence of MSX.[46] The presence of MSX inhibited incorporation of ^{13}N into total amino acids by 93% in the *A. punctatus* association with wild-type *Nostoc*, but only 83% in the association with MSX-resistant *Nostoc*. The statistically significant difference in inhibition by MSX was taken to reflect the amount of N_2-derived NH_4^+ assimilated by symbiotically associated *Nostoc* and was estimated to be on the order of 10 to 20% of the total fixed nitrogen.[46] This result verified that, in association with *A. punctatus*, *Nostoc* sp. strain 7801 does assimilate NH_4^+, but also that its assimilatory capacity is greatly impaired and that NH_4^+ is directly made available for growth of the eukaryotic partner. Stewart and Rodgers[50] previously showed that the *Nostoc* sp. symbiont in *Blasia pusilla* released fixed nitrogen primarily as NH_4^+, but the fraction of nitrogen fixed and subsequently released by the *Nostoc* was not determined. Those experiments were done by incubating manually excised *Nostoc* colonies for 72 h in continuous light and analyzing the medium for NH_4^+, amino acids, and peptides; controls were not taken to insure that the rate of N_2 fixation was similar to that *in situ* or that the measured extracellular products were not from lysed cells.

In experiments analogous to the use of MSX, mutants of *Nostoc* sp. strain 7801 have been isolated that are resistant to the herbicide 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).[45] DCMU blocks noncyclic photosynthetic electron transport in both *Nostoc* and *Anthoceros* by interfering with plastoquinone binding to the D1 protein of the photosystem II reaction center complex.[51] In the presence of DCMU, acetylene reduction by the *A. punctatus* association with wild-type *Nostoc* sp. strain 7801 was inhibited 97%, while that of an association with DCMU-resistant *Nostoc* sp. strain 7801 was inhibited only 69%.[45] This indirect assay of DCMU-insensitive nitrogen fixation by the association with the resistant *Nostoc* was taken as evidence of photosynthetic activity by symbiotically associated *Nostoc* and confirmation of the results of *in vivo* CO_2 fixation by immediately isolated symbiotic *Nostoc* (Table 3).

Regulation of NH_4^+ Assimilation

The lower *in vivo* and *in situ* NH_4^+ assimilation activity of *Nostoc* associated with *A. punctatus* could be a consequence of repressing the synthesis of GS, the initial enzyme of the GOGAT cycle, inhibiting its activity or depriving it of substrate. The results presented in Table 4 show that the specific activity of GS in crude extracts of symbiotically grown *Nostoc* sp. strain 7801 is about threefold lower than the activity in free-living *Nostoc* sp. strain 7801. The difference in specific activity is of the same magnitude as when *in vivo* NH_4^+ assimilation is compared[46] and indicates that substrate limitation of GS is not responsible for the depression in NH_4^+ assimilation by symbiotically associated *Nostoc* sp. strain 7801. Based on results from both quantitative immunoelectrophoresis (rocket gels) and enzyme linked immunosorbent assay (ELISA) experiments,[52,53] the amount of GS protein per soluble cell protein is not statistically different in *Nostoc* sp. strain 7801 from symbiotic or free-living growth conditions (Table 4).[52] The immunological assays indicate regulation of NH_4^+ assimilation by posttranslational control of GS in *Nostoc* sp. strain 7801 associated with *A. punctatus*. Posttranslational control of GS in this association is in contrast to reports

TABLE 4
In Vitro Properties of GS from *Nostoc* sp. Strain 7801

Property	Growth state Free-living	 Symbiotic
Specific catalytic activity[a] (μmol/min/mg protein)	0.13	0.05 (38)
Protein concentration[b] (μg GS/mg cell protein)	7.1	6.1 (86)

Note: Numbers in parenthesis reflect the percent of free-living values.

[a] Specific activity in crude cell extracts is from assays of biosynthetic γ-glutamylhydroxamate formation; means of 5 replicates for each.[53]

[b] Protein concentration was determined by immunoelectrophoresis and enzyme linked immunosorbent assays using GS purified from free-living cultures of *Nostoc* sp. strain 7801 as standard and as antigen in antibody induction in rabbits;[52] means of 7 (free-living) and 2 (symbiotic) replicates.

of decreased synthesis of the enzyme in the symbionts associated with *Azolla*[8,53,54] and certain lichens,[8] and no apparent modification of GS catalytic activity or protein level in *Nostoc* sp. associated with certain cycads.[55] The mechanism of posttranslational modification of GS in *Nostoc* associated with *A. punctatus* is not yet known; it could occur by covalent modification or by sulfhydral oxidation/reduction, which has been shown to occur in cyanobacteria.[56] Western blot analysis indicates that limited proteolysis is not the cause of the lower GS-specific activity in symbiotically associated *Nostoc* sp. strain 7801.[52]

The values of the GS protein concentration in free-living and symbiotic *Nostoc* sp. strain 7801 shown in Table 4 differ from the values published by Joseph and Meeks.[52] The previously published values are high by a factor of 8 because of an undetected error in measurements of the concentration of purified GS in establishment of standard curves for the immunoassays. However, the comparisons between values for free-living and symbiotically grown cultures in both sets of experiments are internally consistent and the conclusion of a posttranslational modification of GS in *Nostoc* grown in association with *A. punctatus*, as opposed to regulation of synthesis, remains valid.

Regulation of CO_2 Assimilation

The demonstrated photosynthetic fixation of CO_2 by *Nostoc* sp. strain 7801 immediately isolated from symbiotic association with *A. punctatus* (Table 3) is in contrast to the results of Rodgers and Stewart;[20] they were unable to detect any light-dependent O_2 evolution by the *Nostoc* sp. immediately isolated from *B. pusilla*. The observation of *in situ* DCMU-insensitive acetylene reduction only by the association with the *Nostoc* sp. strain 7801 DCMU-resistant mutant confirms that the *in vivo* photosynthetic activity measured in *Nostoc* immediately isolated from *A. punctatus* was not due to contamination of the preparations by eukaryotic chloroplasts. In a manner similar to NH_4^+ assimilation, the lower rate of *in vivo* CO_2 fixation by symbiotic *Nostoc* sp. strain 7801, relative to free-living cultures, is paralleled by a lower *in vitro* specific activity of Rubisco (Table 5). Moreover, the amount of Rubisco protein in symbiotic cells is similar to the values found in free-living cultures (Table 5), when one considers that at least 45% of the symbiotic *Nostoc* cells are active or degenerative heterocysts. Heterocysts of free-living cyanobacteria lack the Rubisco protein.[57] Vegetative cells of symbiotically associated *Nostoc* sp. strain 7801 contain carboxysomes, aggregates of Rubisco, at concentrations equivalent to free-living cultures, whereas such structures are not apparent in heterocysts from free-living or symbiotic growth states (Figure 4). Rodgers and Stewart[20] and Duckett et al.[22] also observed the presence of carboxysomes

TABLE 5
In Vitro Properties of Rubisco from *Nostoc* sp. Strain 7801

Property	Growth state	
	Free-living	Symbiotic
Specific catalytic activity[a] (nmol/min/mg protein)	215	25 (12)
Protein concentration[b] (μg Rubisco/mg cell protein)	52	43 (83)

Note: Numbers in parentheses reflect the percent of free-living values.

[a] Specific activity in crude cell extracts from assays of ribulose-1,5-biphosphate-dependent incorporation of $^{14}CO_2$ into acid stable products; values are means of 7 (free-living) and 9 (symbiotic) replicates.[45]

[b] Protein concentration was determined from ELISA using Rubisco purified from free-living cultures of *Nostoc* sp. strain 7801 as standard and Rubisco large subunit as antigen in antibody induction in rabbits; values are means of 14 (free-living) to 22 (symbiotic) replicates for each.

in vegetative cells, but not heterocysts, of *Nostoc* spp. associated with *B. pusilla* and *Anthoceros* species. The results summarized in Table 5 indicate that the activity of Rubisco, like that of GS, is controlled by a posttranslational mechanism and that synthesis of Rubisco by *Nostoc* sp. strain 7801 is not repressed in the symbiotic growth state with *A. punctatus.* The catalytic activity of Rubisco in oxygenic phototrophs can be inhibited on various levels by a number of effectors and physiological states.[58] Although the lower rate of *in vivo* CO_2 fixation by symbiotically associated *Nostoc* sp. 7801 appears not to be singular consequence of inhibition of photochemical reactions, these reactions could also decline in parallel with that of carboxylation.

Symbiotic Nitrogen Fixation

The rate of nitrogen fixation by symbiotic *Nostoc* sp. strain 7801 appears to be strongly dependent on association with intact *A. punctatus* tissue. At low concentrations, digitonin increases the permeability of eukaryotic cells more extensively than it does of prokaryotic cells.[59] Within 60 min of treatment of associated *A. punctatus-Nostoc* sp. strain 7801 with 0.05% (w/v) digitonin, the rates of acetylene reduction and $[^{13}N]N_2$ fixation decline to about 25% and 15%, respectively, of the control rates of intact tissue; the rates of acetylene reduction recover to about 50% of the control values after 3 h of continual exposure to digitonin.[60] Control experiments showed that digitonin (0.05%, w/v) had no effect on $^{13}NH_4^+$ assimilation by free-living or symbiotic *Nostoc* sp. strain 7801.[48] This dependence upon the intact association for maximal nitrogen fixation activity precludes direct determination of nitrogenase-specific activities or quantitation of the amount of fixed nitrogen excreted by immediately isolated symbiotic *Nostoc.*

Attempts have been made to quantitate the muramic acid content of the *A. punctatus-Nostoc* sp. strain 7801 association so that *in situ* metabolic activities could be normalized to a parameter of biomass unique to the cyanobacterium.[61] Unfortunately, current methods of muramic acid extraction and analysis (including high-performance liquid chromatography) are too insensitive for accurate determinations with manageable amounts of associated bryophyte tissue. However, *in situ* rates of acetylene reduction have been normalized to cyanobacterial protein in the *B. pusilla*[50] and *A. punctatus* associations.[45] Stewart and Rodgers[50] attempted to physically recover, by manual extrusion, all of the symbiotic *Nostoc* from *B. pusilla* samples for protein determination after measurements of acetylene reduction. Based on this experimental approach, Rodgers and Stewart[50] reported specific activities of symbiotic acetylene reduction of 70 nmol of C_2H_2 reduced per milligram of *Nostoc* protein per hour, a value 3.2-fold higher than their free-living cultures. The mean protein content per colony of *Nostoc* sp. strain 7801 has been determined as 0.55 ± 0.04 μg (mean ± SE of 1511 colonies from 7 isolations) when immediately isolated from *A. punctatus* tissue of standard low-light-grown physiological state (age after reconstitution and time after penicillin treatment).[45] Using *A. punctatus-Nostoc* sp. strain 7801 cultures of the identical physiological state used above to determine protein content, specific activities of 1344 nmol C_2H_2 reduced per milligram *Nostoc* protein per hour were measured in comparison to 378 nmol/mg protein per hour in free-living cultures of *Nostoc* sp. strain 7801, a 3.6-fold difference (Table 3).[45] Although the absolute rates of acetylene reduction differ in the two studies, the fold increase in symbiotic relative to free-living activities is of similar magnitude. These two studies provide evidence of a substantial increase in cyanobacterial nitrogenase-specific activity as a consequence of symbiotic association and the increase corresponds to the increase in heterocyst frequency.

In the reduction of N_2 to NH_4^+, the nitrogenase enzyme complex (dinitrogenase reductase and dinitrogenase) requires ATP as well as an electron donor of low redox potential. In free-living cyanobacteria, the reductant necessary for nitrogenase activity is thought to be derived from photosynthate in the form of hexoses supplied from adjacent vegetative cells and the ATP provided by cyclic photophosphorylation.[62] Because the *Nostoc* symbiont in gametophyte tissues of *A. punctatus* and *B. pusilla* from field populations apparently lacked complete photosynthetic activity, Stewart and Rodgers[50] proposed that the source of reductant for nitrogenase activity in these associations must come from host eukaryotic tissue. The proof of a host photosynthesis-dependent nitrogenase activity was obtained from well-conceived acetylene reduction experiments in which the *Nostoc* colonies and gametophyte tissue of *A. punctatus* was darkened by a layer of fine charcoal and the sporophytes exposed to light. Little or no acetylene was reduced in these experiments if the sporophytes were also darkened or excised from the gametophytes.[50]

Under pure liquid culture conditions, *Nostoc* sp. strain 7801 in association with *A. punctatus* has a demonstrable rate of photosynthetic CO_2 fixation (Table 3). The observation that only DCMU-resistant mutants of *Nostoc* sp. strain 7801 show DCMU-insensitive acetylene reduction when in association with *A. punctatus* is evidence that the *Nostoc* can directly supply photosynthate as a source of reductant for its nitrogenase activity. When *A. punctatus* associations with wild-type *Nostoc* sp. strain 7801 are exposed to DCMU and then supplemented with carbohydrates such as glucose, fructose, or sucrose at 100 m*M*, 87% of the acetylene reduction rate of uninhibited cultures is obtained (Table 6).[45] Furthermore, 65% of the light-dependent rate of acetylene reduction can be measured in dark-incubated cultures of *A. punctatus-Nostoc* sp. strain 7801 in the additional presence of 100 m*M* glucose, fructose, or sucrose. These results are evidence of symbiotic nitrogen fixation dependent upon photoheterotrophic and dark heterotrophic metabolism, respectively. In neither set of experiments could the stimulation by carbohydrates be duplicated with dicarboxylic acids.[45] The general conclusion from these experiments is that symbiotic *Nostoc* sp. strain 7801 directly maintains a pool of photosynthate in vegetative cells that supports nitrogenase activity in heterocysts. The pool of photosynthate most probably is supplemented by extracellular products of *Anthoceros* metabolism. The identity of the extracellular products that stimulate nitrogen fixation has not been determined; they are presumably carbohydrate. Based on a preliminary experiment involving lengthy (7 d) incubation of symbiont-free *B. pusilla* in the presence of $^{14}CO_2$ followed by paper chromatography of the culture medium, Stewart and Rodgers[50] suggested that sucrose was the extracellular compound transported from bryophyte to cyanobacterium.

Regulation of Heterocyst Differentiation

The original observations by Fogg,[63] showing an absence of heterocysts in cultures grown in the presence of NH_4^+, forms the basis of the general hypothesis of many researchers

TABLE 6

Rates of Symbiotic Nitrogen Fixation by *Nostoc* sp. Strain 7801 Dependent on Photoautotrophic, Photoheterotrophic, and Dark Heterotrophic Metabolism

	Rate of C_2H_2 reduction and incubation condition[a]				
Strain in association	Light − DCMU	Light + DCMU	Light + DCMU + 100 m*M* glucose[b]	Dark	Dark + 100 m*M* glucose
Wild-type	9.43	0.28 (3)	8.20 (87)	0.08 (.01)	6.10 (65)
DCMU-resistant	9.43	2.89 (31)	—	—	—

Note: Numbers in parentheses reflect the percent of light-dependent, minus DCMU, values.

[a] Rate is nmole of C_2H_2 reduced/min/g fresh weight of associated *Anthoceros punctatus-Nostoc* sp. strain 7801 tissue; the control rate (light minus DCMU) was independent of the strain in association; values are means of 5 to 13 replicates.[45]

[b] Values are given for inclusion of glucose in photoheterotrophic or dark heterotrophic assays; identical rates were obtained with fructose and sucrose; maltose, 30 m*M* malate, succinate, or citrate were ineffective in restoring the control light-dependent rate of C_2H_2 reduction.

that regulation of heterocyst differentiation in free-living cyanobacteria involves a type of nitrogen control mechanism functioning through a nitrogen catabolite repressor. Nitrogen control refers to the elevation or repression of the synthesis of enzyme systems involved in the assimilation, or generation, of NH_4^+ and formation of glutamate for cellular growth.[64] A model of nitrogen control that is pleiotrophic for a number of enzymes, including nitrogenase, has been proposed and is being tested genetically and biochemically in enteric bacteria.[64] Although an analogous system may function in heterocyst-forming cyanobacteria, there is yet no direct experimental evidence.

Three general properties of *Nostoc* sp. strain 7801 in association with *A. punctatus* imply that the regulation of heterocyst differtiation and nitrogenase expression in symbiosis is different from that operative in the free-living state and may not directly involve a nitrogen catabolite repressor. The first property concerns coordinated expression of GS, nitrogenase, and heterocyst formation. In *Anabaena* sp. strain PCC 7120, the structural genes for nitrogenase *(nif)* and GS *(glnA)* share a common promotor sequence.[65] As a consequence, elevation of the synthesis of nitrogenase in free-living cyanobacterial cultures is accompanied by an increase in GS synthesis.[52,65] However, the 3.6-fold increased expression of nitrogenase in symbiotic *Nostoc* sp. strain 7801 relative to free-living cultures (Table 3) is not accompanied by increased synthesis of GS (Table 4). In addition, inhibition of GS activity by MSX allows for differentiation of heterocysts and expression of nitrogenase activity in the presence of NH_4^+.[66,67] This observation led to the idea that the high heterocyst frequency in symbiotic cyanobacteria may be a consequence of decreased GS activity.[8,68] Treatment of *A. punctatus-Nostoc* sp. strain 7801 cultures with NH_4^+ results in a decline in both nitrogenase activity and heterocyst frequency.[23] However, *in vivo* NH_4^+ assimilation (Table 3) and the specific activity of GS, as well as amount of GS protein, are similar in *Nostoc* sp. strain 7801 immediately isolated from N_2- or NH_4^+-grown symbiotic associations.[52] Thus, it would appear that GS and its regulation play no direct role in heterocyst differentiation under symbiotic growth conditions.

Second, the observation that exogenous NH_4^+ does result in a repression or lack of stimulation of heterocyst differentiation in symbiotically associated *Nostoc* sp. strain 7801 implies a repressive mechanism similar to free-living cultures.[23] However, *in situ*, symbiotic *Nostoc* sp. strain 7801 excretes from 80 to 90% of its fixed nitrogen as NH_4^+;[46] increasing the amount of NH_4^+ by exogenous supply should not alter the extent to which *Nostoc* may metabolize it. By default, the exogenous NH_4^+ must be metabolized by *A. punctatus*, effecting a chain of currently unknown events that ceases the stimulation of heterocyst differentiation by associated *Nostoc*.

Third, neither vegetative cells nor heterocysts of symbiotically grown *Nostoc* sp. strain 7801 have the characteristics of nitrogen-limited or -starved cells. Vegetative cells of free-living, N_2-grown cyanobacteria contain few or no cyanophycin granules, the unique nitrogen storage polymer of cyanobacteria; such granules are in high density in NH_4^+-grown cultures and are degraded during nitrogen deprivation.[44] Vegetative cells of *Nostoc* sp. strain 7801 in association with *A. punctatus* contain numerous cyanophycin granules (Figure 4A). Phycobiliproteins are degraded as an immediate consequence of nitrogen deprivation in free-living cyanobacteria;[69] thus the vegetative cell that differentiates into a heterocyst lacks the pigments or loses them early during differentiation (Figure 3B). However, the presence of phycobiliproteins in heterocysts and coupling of their light absorbance to nitrogenase activity has been known for some time in the cyanobacterium associated with the water fern *Azolla*.[70] By epifluorescence microscopy using 560 nm exciting light (absorbed by phycoerythrin) and >600 nm emission, it can be seen that more than 90% of *A. punctatus*-associated *Nostoc* sp. strain 7801 cells fluoresce (Figure 3C and D) and, by electron microscopy, about 45% of those cells are active and degenerative heterocysts.[42] There are no indications that the phycobiliproteins present in heterocysts of symbiotic *Nostoc* sp. strain 7801 are organized into phycobilisomes (Figure 4) or are coupled to nitrogenase activity. Nevertheless, it would appear that a nitrogen starvation signal may not be involved in symbiotic heterocyst differentiation in the hornwort association.

A symbiotic regulatory system, distinct from the enteric nitrogen regulatory system, has been suggested in Rhizobial bacteriods to partially explain uncoupled *nif* and *glnA* transcription in the symbiotic state.[71] An analogous symbiotic regulatory system for heterocyst differentiation and nitrogenase expression may occur in the *A. punctatus-Nostoc* sp. strain 7801 association. The system need not to have evolved specifically in symbiosis; rather its unique expression in *Nostoc* could come from *Anthoceros*-dependent activation of an existing system in an unusual growth condition.

SUMMARY

To recapitulate, an exploitable characteristic of the *A. punctatus* symbiotic association with *Nostoc* spp. for basic research is the ease and flexibility of experimental manipulations. The only disadvantage of this association is the difficulty in obtaining large quantities of symbiotically associated *Nostoc* spp. biomass for biochemical assays, especially in comparisons to the *Azolla* association. Nevertheless, as summarized in this review, by using sensitive and low volume assays, certain physiological properties of the symbiotically associated *Nostoc* have been defined. For example, an extensive uncoupling between the reduction of dinitrogen and the assimilation of the reduced product, ammonium, apparently occurs in the cells of symbiotically associated *Nostoc* sp. strain 7801 and, no doubt, in other cyanobacterial symbiotic associations. The uncoupling in symbiosis is exaggerated by both the increased rate of N_2 fixation and decreased rate of NH_4^+ assimilation. This uncoupling implies first that the flux of carbon in providing reductant for nitrogenase must be higher in symbiotic than free-living cells and, second, that metabolic pathways of carbon catabolism may be altered in symbiosis so that the substrate for NH_4^+ assimilation, α-ketoglutarate, is produced only at levels comparable to the low rates of assimilation. Thus, one predicts the presence and expression in symbiotically associated cyanobacteria of regulatory systems of intermediary carbon metabolism in addition to those influencing inorganic carbon and nitrogen assimilation and cellular differentiation. The inability to demonstrate alteration in the level of enzymes of intermediary metabolism initially led to the idea that cyanobacteria lack mechanisms for regulation of gene transcription.[72] The symbiotic state may represent the physiological growth conditions where specific cyanobacterial genetic regulatory systems will be identified and characterized.

A major hindrance in identifying unique regulatory mechanisms or systems in N_2-fixing cyanobacteria has been the inability to biologically complement mutations by experimentally controlled transfer of DNA between recipient and donor cells. The complementation barrier has been all but eliminated by the efforts of Wolk and colleagues in the construction of basic shuttle vectors capable of replication in *Escherichia coli* and certain strains of *Anabaena* and *Nostoc*.[73,74] These vectors can be conjugated from *E. coli* into a number of *Nostoc* strains that are both symbiotically competent and incompetent in the *A. punctatus* association.[75] In addition to direct physiological and biochemical characterization, this laboratory now uses the *A. punctatus* symbiotic association to analyze the phenotype, and their complementation in trans, of spontaneous and chemically generated *Nostoc* mutants with specific alterations in formation of infective units (hormogonia), infection of host tissue, and heterocyst differentiation and nitrogen fixation in the free-living state.

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REFERENCES

1. **Schofield, W. B.,** *Introduction to Bryology,* Macmillan, New York, 1985.
2. **Katoh, K., Hori, H., and Osawa, S.,** The nucleotide sequences of 5S ribosomal RNAs from four Bryophyta-species, *Nucleic Acids Res.,* 11, 5671, 1983.
3. **Clymo, R. S. and Hayward, P. M.,** The ecology of *Sphagnum,* in *Bryophyte Ecology,* Smith, A. J. E., Ed., Chapman and Hall Ltd., London, 1982, 229.
4. **Bond, G. and Scott, G. D.,** An examination of some symbiotic systems for fixation of nitrogen, *Ann. Bot.,* 19, 67, 1955.
5. **Rodgers, A.,** The effect of some external factors on nitrogenase activity in the free-living and endophytic *Nostoc* of the liverwort *Blasia pusilla, Physiol. Plant.,* 44, 407, 1978.
6. **Sprent, J. I. and Raven, J. A.,** Evolution of nitrogen-fixing symbioses, *Proc. Soc. Edinburgh,* 85B, 215, 1985.
7. **Millbank, J. W.,** Associations with blue-green algae, in *The Biology of Nitrogen Fixation,* Quispel, A., Ed., American Elsevier, New York, 1974, 238.
8. **Stewart, W. D. P., Rowell, P., and Rai, A. N.,** Symbiotic nitrogen-fixing cyanobacteria, in *Nitrogen Fixation,* Stewart, W. D. P. and Gallon, J. R., Eds., Academic Press, New York, 1980, 239.
9. **Stewart, W. D. P.,** *Nitrogen Fixation in Plants,* Athlone Press, London, 1966.
10. **Granhall, U. and Selander, H.,** Nitrogen fixation in a subarctic mire, *Oikos,* 24, 8, 1973.
11. **Granhall, U. and von Hofsten, A.,** Nitrogenase activity in relation to intracellular organisms in *Sphagnum* mosses, *Physiol. Plant.,* 36, 88, 1976.
12. **Brasilier, K., Granhall, U., and Stenstrom, T.-A.,** Nitrogen fixation in wet minerotrophic moss communities of a subarctic mire, *Oikos,* 31, 236, 1978.
13. **Alexander, V. and Schell, D. M.,** Seasonal and spatial variation of nitrogen fixation in the Barrow, Alaska, tundra, *Arct. Alp. Res.,* 5, 77, 1973.
14. **Brasell, H. M., Davies, S. K., and Mattay, J. P.,** Nitrogen fixation associated with bryophytes colonizing burnt sites in Southern Tasmania, Australia, *J. Bryol.,* 14, 139, 1986.
15. **Vlassak, K., Paul, E. A., and Harris, R. E.,** Assessment of biological nitrogen fixation in grassland and associated sites, *Plant Soil,* 38, 637, 1973.
16. **Scheirer, D. C. and Brasell, H. M.,** Epifluorescence microscopy for the study of nitrogen fixing blue-green algae associated with *Funaria hygrometrica* (Bryophyta), *Am. J. Bot.,* 71, 461, 1984.
17. **Reddy, G. B. and Giddens, J.,** Nitrogen fixation by moss-algal association in grassland, *Soil Biol. Biochem.,* 13, 537, 1981.

18. **Watanabe, A. and Kiyohara, T.**, Symbiotic blue-green algae of lichens, liverworts and cycads, in *Studies on Microalgae and Photosynthetic Bacteria*, Japanese Society of Plant Physiologists, Eds., University of Tokyo Press, Tokyo, 1973, 189.
19. **Ridgway, J. E.**, The biotic relationship of *Anthoceros* and *Phaeoceros* to certain cyanobacteria, *Ann. Mo. Bot. Gard.*, 54, 95, 1967.
20. **Rodgers, G. A. and Stewart, W. D. P.**, The cyanophyte-hepatic symbiosis. I. Morphology and physiology, *New Phytol.*, 78, 441, 1977.
21. **Dalton, D. A. and Chatfield, J. M.**, A new nitrogen-fixing cyanophyte-hepatic association: *Nostoc* and *Porella*, *Am. J. Bot.*, 72, 781, 1985.
22. **Duckett, J. G., Prasad, A. K. S. K., Davies, D. A., and Walker, S.**, A cytological analysis of the *Nostoc*-bryophyte relationship, *New Phytol.*, 79, 349, 1977.
23. **Enderlin, C. S. and Meeks, J. C.**, Pure culture and reconstitution of the *Anthoceros-Nostoc* symbiotic association, *Planta*, 158, 157, 1983.
24. **Silvester, W. B.**, Dinitrogen fixation by plant associations excluding legumes, in *A Treatise in Dinitrogen Fixation*, Vol. 4, Hardy, R. W. F. and Gibson, A. H., Eds., John Wiley & Sons, New York, 1977, 141.
25. **Brock, T. D.**, Evolutionary and ecological aspects of the cyanophytes, in *The Biology of Blue-Green Algae*, Carr, N. G. and Whitton, B. A., Eds., Blackwell Scientific, Oxford, 1973, 487.
26. **Basilier, K.**, Fixation and uptake of nitrogen in *Sphagnum* blue-green algal associations, *Oikos*, 34, 239, 1980.
27. **Granhall, U.**, Nitrogen fixation by blue-green algae in temperate soils, in *Nitrogen Fixation by Free-Living Microorganisms*, Stewart, W. D. P., Ed., Cambridge University Press, Cambridge, 1975, 189.
28. **Granhall, U. and Lindberg, T.**, Nitrogen fixation in some coniferous forest ecosystems, *Ecol. Bull. (Stockholm)*, 26, 178, 1978.
29. **Doyle, W. T.**, Mosses and liverworts, in *Methods in Developmental Biology*, Wilt, F. H. and Wessells, N. K., Eds., T. Y. Crowell, New York, 1967, 329.
30. **Matzke, E. B.**, The aseptic culture of liverworts in microphytotrons, *Bryologist*, 67, 136, 1964.
31. **Basile, D. V.**, A comparison of some macronutrient media used to culture bryophytes, *The Bryologist*, 78, 403, 1975.
32. **Michalis, L. and Doyle, W. T.**, Submerged growth of pure cultures of the liverwort *Sphaerocarpos donnellii*, *Physiol. Plant.*, 15, 351, 1962.
33. **Meeks, J. C.**, Symbiotic associations, *Methods Enzymol.*, 167, 113, 1988.
34. **Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y.**, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J. Gen. Microbiol.*, 111, 1, 1979.
35. **Campbell, E. L. and Meeks, J. C.**, Characteristics of hormogonia formation by symbiotic *Nostoc* spp. in response to the presence of *Anthoceros punctatus* or its extracellular products, *Appl. Environ. Microbiol.*, 55, 125, 1989.
36. **Dow, C. S., Whittenbury, R., and Carr, N. G.**, The "shut down" or "growth precursor" cell — an adaptation for survival in a potentially hostile environment, in *Microbes in Their Natural Environment*, Slater, J. H., Whittenbury, R., and Wimpenny, J. W. T., Eds., Cambridge University Press, Cambridge, 1983, 187.
37. **Lachance, M.-A.**, Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassociation, *Int. J. Syst. Bacteriol.*, 31, 139, 1981.
38. **Newton, J. W. and Herman, A. I.**, Isolation of cyanobacteria from the aquatic fern, *Azolla*, *Arch. Microbiol.*, 120, 161, 1979.
39. **Meeks, J. C., Joseph, C. M., and Haselkorn, R.**, Organization of the *nif* genes in cyanobacteria in symbiotic association with *Azolla* and *Anthoceros*, *Arch. Microbiol.*, 150, 61, 1988.
40. **Pankow, H. and Marten, B.**, Über *Nostoc sphaericum* Vach., *Arch. Mikrobiol.*, 48, 203, 1964.
41. **Denarie, J. G., Truchet, G., and Bergeron, B.**, Effects of some mutations of symbiotic properties of *Rhizobium*, in *Symbiotic Nitrogen Fixation in Plants*, Nutman, P. S., Ed., Cambridge University Press, Cambridge, 1976, 47.
42. **Weeden, Y. M. and Meeks, J. C.**, unpublished observations, 1987.
43. **Adams, D. G. and Carr, N. G.**, The development biology of heterocyst and akinete formation in cyanobacteria, *Crit. Rev. Microbiol.*, 38, 1, 1984.
44. **Allen, M. M.**, Cyanobacterial cell inclusions, *Annu. Rev. Microbiol.*, 38, 1, 1984.
45. **Steinberg, N. A. and Meeks, J. C.**, unpublished observations, 1988.
46. **Meeks, J. C., Enderlin, C. S., Joseph, C. M., Chapman, J. S., and Lollar, M. W. L.**, Fixation of $[^{13}N]N_2$ and transfer of fixed nitrogen in the *Anthoceros-Nostoc* association, *Planta*, 164, 406, 1985.
47. **Meeks, J. C.**, Radiotracer studies of nitrogen assimilation in cyanobacteria, *Recent Adv. Chem.*, 197, 269, 1981.
48. **Meeks, J. C., Enderlin, C. S., Wycoff, K. L., Chapman, J. S., and Joseph, C. M.**, Assimilation of $^{13}NH_4^+$ by *Anthoceros* grown with and without symbiotic *Nostoc*, *Planta*, 158, 384, 1983.

49. **Ronzio, R., Rowe, W., and Meister, A.,** Studies on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine, *Biochemistry,* 8, 1066, 1969.
50. **Stewart, W. D. P. and Rodgers, G. A.,** The cyanophyte-hepatic symbiosis. II. Nitrogen fixation and the interchange of nitrogen and carbon, *New Phytol.,* 78, 459, 1977.
51. **Bryant, D. A.,** The cyanobacterial photosynthetic apparatus: comparison of those of higher plants and photosynthetic bacteria, *Can. Bull. Fish. Aquatic Sci.,* 214, 423, 1987.
52. **Joseph, C. M. and Meeks, J. C.,** Regulation of expression of glutamine synthetase in a symbiotic *Nostoc* strain associated with *Anthoceros punctatus, J. Bacteriol.,* 169, 2471, 1987.
53. **Lee, K. Y., Joseph, C. M., and Meeks, J. C.,** Glutamine synthetase specific activity and protein concentration in symbiotic *Anabaena* associated with *Azolla caroliniana, Antonie van Leewenhoek,* 54, 345, 1988.
54. **Orr, J. and Haselkorn, R.,** Regulation of glutamine synthetase activity and synthesis in free-living and symbiotic *Anabaena* spp., *J. Bacteriol.,* 152, 626, 1982.
55. **Lindblad, P. and Bergman, B.,** Glutamine synthetase: activity and localization in cyanobacteria of the cycads *Cycas revoluta* and *Zamia skinneri, Planta,* 169, 1, 1986.
56. **Papen, H. and Bothe, J.,** The activation of glutamine synthetase from the cyanobacterium *Anabaena cylindrica* by thioredoxin, *FEMS Microbiol. Lett.,* 23, 41, 1984.
57. **Codd, G. A. and Stewart, W. D. P.,** Ribulose 1,5-bisphosphate carboxylase in heterocysts and vegetative cells of *Anabaena cylindrica, FEMS Microbiol. Lett.,* 2, 247, 1977.
58. **Miziorko, H. M. and Lorimer, G. H.,** Ribulose-1,5-bisphosphate carboxylase-oxygenase, *Annu. Rev. Biochem.,* 52, 507, 1983.
59. **Chambers, S., Morris, M., and Smith, D. C.,** Lichen physiology. XV. The effect of digitoxin and other treatments on biotrophic transport of glucose from alga to fungus in *Peltigera polydactyla, New Phytol.,* 76, 485, 1976.
60. **Meeks, J. C., Enderlin, C. S., Joseph, C. M., Steinberg, N. A., and Weeden, Y. M.,** Use of ^{13}N to study N_2 fixation and assimilation by cyanobacterial-lower plant associations, in *Nitrogen Fixation Research Progress,* Evans, H. J., Bottomley, P. J., and Newton, W. E., Eds., Martinus Nijhoff, Dordrecht, Netherlands, 1985, 301.
61. **Enderlin, C. S.,** Pure Culture, Reconstitution, and Physiology of the Symbiotic Association Between the Bryophyte *Anthoceros* and the Cyanobacterium *Nostoc,* Ph.D. thesis, University of California, Davis, 1986.
62. **Wolk, C. P.,** Heterocysts, in *The Biology of Cyanobacteria,* Carr, N. G., and Whitton, B. A., Eds., Blackwell Scientific, Oxford, 1982, 359.
63. **Fogg, G. E.,** Growth and heterocyst production in *Anabaena cylindrica* Lemm. II. In relation to carbon and nitrogen metabolism, *Ann. Bot. (London),* 13, 241, 1949.
64. **Kustu, S., Sei, K., and Keener, J.,** Nitrogen regulation in enteric bacteria, in *Regulation of Gene Expression — 25 Years On,* Soc. Gen. Microbiol., Symp. 39, Booth, I. R. and Higgins, C. F., Eds., Cambridge University Press, 1986, 139.
65. **Tumer, N. E., Robinson, S. J., and Haselkorn, R.,** Different promoters for the *Anabaena* glutamine synthetase gene during growth using molecular or fixed nitrogen, *Nature (London),* 306, 337, 1983.
66. **Stewart, W. D. P. and Rowell, P.,** Effects of *L*-methionine-DL-sulfoximine on the assimilation of newly fixed NH_3, acetylene reduction, and heterocyst production in *Anabaena cylindrica, Biochem. Biophys. Res. Commun.,* 65, 846, 1977.
67. **Ownby, J. D.,** Effects of amino acids on the methionine-sulfoximine-induced heterocyst formation in *Anabaena, Planta,* 136, 277, 1977.
68. **Haselkorn, R.,** Heterocysts, *Annu. Rev. Plant Physiol.,* 29, 319, 1978.
69. **Wood, N. B. and Haselkorn, R.,** Control of phycobiliprotein proteolysis and heterocyst differentiation in *Anabaena, J. Bacteriol.,* 141, 1375, 1980.
70. **Kaplan, D., Calvert, H. E., and Peters, G. A.,** The *Azolla-Anabaena azollae* relationship. XII. Nitrogenase activity and phycobiliproteins of the endophyte as a function of leaf age and cell type, *Plant Physiol.,* 80, 884, 1986.
71. **Ausubel, F. M., Buikema, W. J., Earl, C. D., Klingensmith, J. A., Nixon, B. T., and Szeto, W. W.,** Organization and regulation of *Rhizobium meliloti* and *Parasponia bradyrhizobium* nitrogen fixation genes, in *Nitrogen Fixation Research Progress,* Evans, H. J., Bottomley, P. J., and Newton, W. E., Eds., Martinus Nijhoff, Dordrecht, Netherlands, 1985, 95.
72. **Carr, N. G.,** Metabolic control and autotrophic physiology, in *The Biology of Blue-Green Algae,* Carr, N. G. and Whitton, B. A., Eds., Blackwell Scientific, Oxford, 1973, 39.
73. **Wolk, C. P., Vonshank, A., Kehoe, P., and Elhai, J.,** Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen fixing filamentous cyanobacteria, *Proc. Natl. Acad. Sci. U.S.A.,* 81, 1561, 1984.
74. **Flores, E. and Wolk, C. P.,** Identification of facultative heterotrophic, N_2 fixing cyanobacteria able to receive plasmid vectors from *Escherichia coli* by conjugation, *J. Bacteriol.,* 162, 1339, 1985.
75. **Wallis, J. and Meeks, J. C.,** unpublished observations, 1988.

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Chapter 4

AZOLLA-ANABAENA SYMBIOSIS: BIOCHEMISTRY, PHYSIOLOGY, ULTRASTRUCTURE, AND MOLECULAR BIOLOGY

Ellen B. Braun-Howland and Sandra A. Nierzwicki-Bauer

INTRODUCTION

The *Azolla-Anabaena* symbiosis is a mutualistic association between the eukaryotic water fern *Azolla* and the prokaryotic, nitrogen-fixing cyanobacterium *Anabaena*. This association has gained attention in recent years because it may represent a viable alternative to chemical nitrogen fertilizers. This and the following chapter summarize the currently available literature on the *Azolla-Anabaena* symbiosis. In this chapter, emphasis is placed on areas of research which have not been reviewed recently. These include studies involving recognition between the two partners, the characterization of endosymbiotic bacteria, and advances in the field of molecular biology. Recent results concerning the sexual hybridization between heterologous *Azolla* species and the reinfection of symbiont-free *Azolla* megasporocarps with *Anabaena azollae* are also addressed.

MORPHOLOGY AND ULTRASTRUCTURE

MORPHOLOGY OF *AZOLLA* SPOROPHYTES

The vegetative characteristics of the water fern *Azolla* change considerably with growth conditions.[1-3] In general, *Azolla* sporophytes (diploid generation of plants) are between 0.5 and 7 cm in diameter and of triangular to polygonal shape.[1] The exception to this is *A. nilotica*, which is elongated and can reach 40 cm or more in length.[4] The main stem (rhizome) of *Azolla* is extensively branched and typically floats prostrate on the surface of water. Under crowded growth conditions, however, some strains of *A. nilotica*, *A. microphylla*, and *A. filiculoides* may exhibit a so-called "mature" morphology, where protions of the sporophyte grow in the vertical direction.[1,4]

An *A. caroliniana* sporophyte grown under laboratory conditions is shown in Figure 1A. In general, laboratory cultures of *Azolla* are between 1 and 3 cm in diameter.[5] The rhizome and branches of *Azolla* bear alternate, deeply bilobed leaves which may overlap. Shoot apices, which are located at each branch tip, curve upwards and out of the water. The leaf trace originates in the stem and branches before entering each leaf. One branch enters the ventral lobe, whereas the other enters the dorsal lobe and partially surrounds the leaf cavity.[6,7]

Adventitious roots arise from the ventral side of the stem at branch points and are chlorophyllous.[1,6,7] In most species, roots are between 1 and 5 cm long, although *A. nilotica* may produce root bundles which are 15 cm or more in length.[1,4] Mature portions of the root bear numerous root hairs, whereas the younger portions and the root apex are protected by a sheath and cap. For a detailed description of root development and anatomy, the reader is referred to Konar and Kapoor,[6] Lumpkin and Plucknett[1] or Gunning et al.[8]

Each lateral leaf comprises a dorsal and ventral lobe which are approximately 1 mm in length. Ventral leaf lobes may be slightly larger than their partner dorsal lobes. The thin, convex ventral lobes are transparent, mostly achlorophyllous, and usually float on the water surface, although they are sometimes partially submerged. The dorsal lobes are aerial, chlorophyllous, and bear papillae of one or more cells. In some instances, characterization

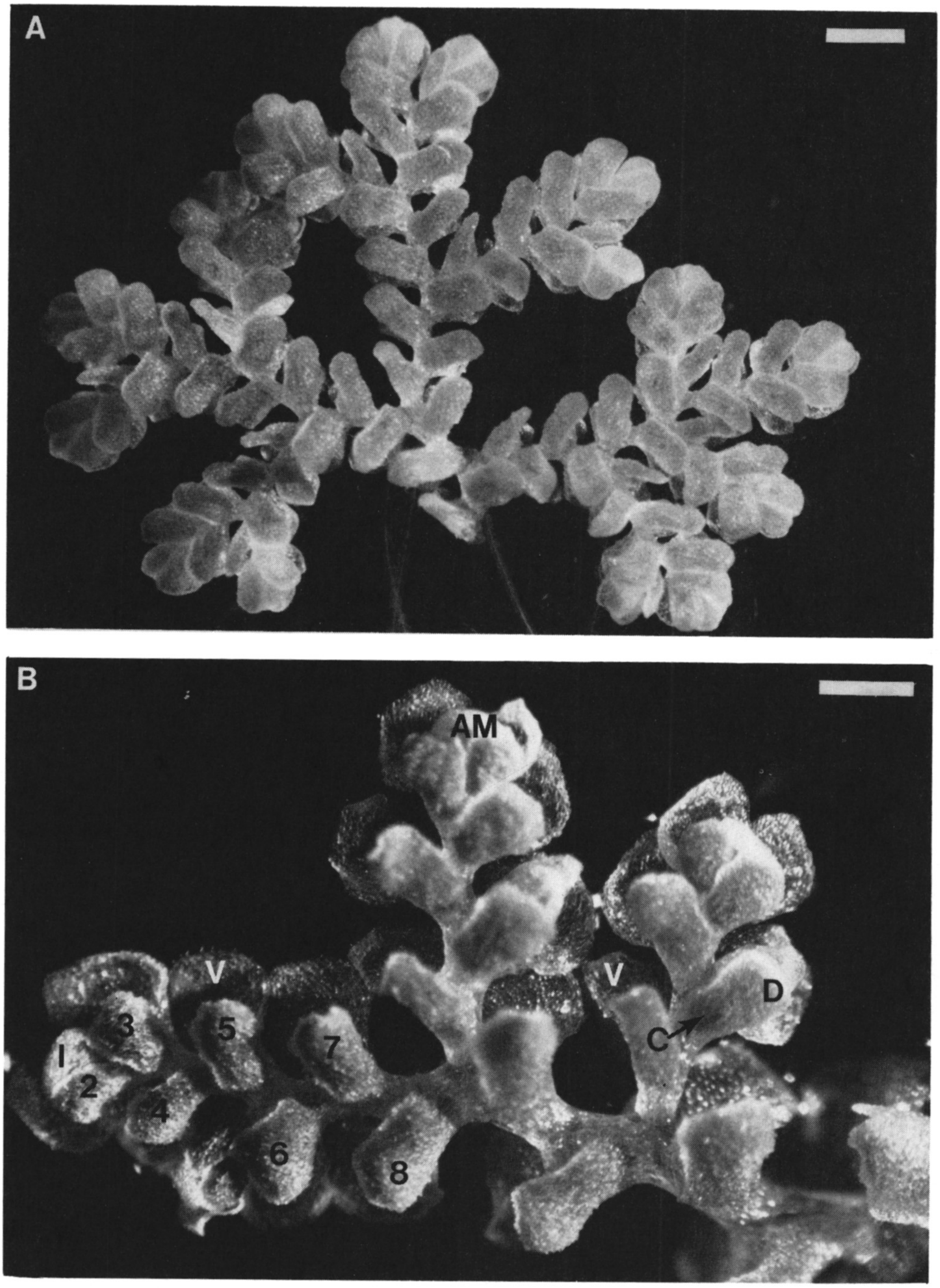


FIGURE 1. Vegetative morphology of *Azolla*. (A) An *A. caroliniana* sporophyte grown under laboratory conditions, illustrating the general morphological characteristics of species belonging to section *Azolla*. Cultures were maintained at 26°C in Fernbach flasks (2.8 l) containing 1 l of IRRI medium, pH 5.5 to 6.0. Flasks were covered with cotton gauze such that free exchange of gases was allowed. Cultures were grown under a 16/8 h (light/dark) photoperiod at a mixed fluorescent and incandescent light intensity of 5 to 8 klx. Bar = 1.5 mm. (B) A higher magnification micrograph of an *A. mexicana* sporophyte. The aerial dorsal leaf lobes (D) contain an extracellular cavity (C) which is normally populated by the cyanobacterium *Anabaena azollae*. Leaves are numbered sequentially along the main rhizome, beginning with the youngest leaves under the apical meristem (AM). The transparent nature of the ventral leaf lobes (V) is clearly illustrated. Bar = 1.5 mm.

of these papillae may facilitate the identification of *Azolla* sporophytes.[1,3] The thin ventral leaf lobes and their location with respect to the thicker dorsal lobes are shown in Figure 1B. Within the dorsal leaf lobe is a specialized cavity that normally contains the cyanobacterium *Anabaena azollae*. When mature, these cavities are ellipsoid in shape, measuring approximately 0.15 × 0.3 mm.[7]

Morphology of Leaf Cavities

A light micrograph of a thick section through a mature *A. caroliniana* leaf cavity is shown in Figure 2. The specialized cavity is extracellularly located in the proximal half of the ventral side of the dorsal leaf lobe. Numerous heterocystous cyanobacterial filaments line the periphery of the cavity wall. Heterocysts are lighter in color than vegetative cells because this section was stained with toluidine blue. This technique has been shown to result in the differential staining of these two cell types.[9] Several highly vacuolated, club-shaped structures, called simple hairs, can also be seen around the cavity periphery.

A scanning electron micrograph (SEM) of an *A. filiculoides* leaf cavity is shown in Figure 3. As in the previous micrograph, cyanobacterial filaments are found in close association with the cavity wall. This peripheral distribution of *Anabaena azollae* is typical of older leaf cavities. In younger leaves, filaments are usually dispersed throughout the cavity.

The development of leaf cavities and hair cells proceeds normally in the absence of the endophyte.[10-13] Isolated leaf cavities, often referred to as algal packets, can be obtained by digesting *Azolla* leaves with a combination of cellulytic enzymes.[7,13] Algal packets are limited by an envelope (10 to 20 nm thick[7,13]) that is rich in fucose[14] and insensitive to digestion with cellulases, proteases, pectinases, or lipases.[7,13] This envelope is apparently of plant origin because it has been isolated from endophyte-free cultures of *Azolla*.[13]

When algal packets are ruptured, cyanobacteria and their associated microflora are released in a mucilagenous matrix[7] that is rich in amino acids and ammonia.[15] The production of mucilage has been ascribed to leaf cavity hairs,[7,16] cyanobacteria,[11,17] and bacteria.[18] However, in contrast to earlier reports,[11] recent results[13] indicate that mucilage is also present in endophyte-free plants. This finding strongly suggests that the mucilage is of plant origin.

A pore opening into algal packets has been occasionally noted in SEM studies, but its function has not been ascertained.[7] The pore may permit gas exchange between the leaf cavity and the atmosphere.[1] If envelope formation occurs during cavity enclosure, it is possible that this pore is homologous to the residual pore located at the top of the cavity where the epidermal cells meet (see below).

Developmental Morphology of the Symbiosis

The growth and development of endosymbiotic *Anabaena azollae* are greatly influenced by the host fern. The growth rate of the endosymbiont is such that it never outgrows the fern, thereby assuring maintenance of the symbiosis.[26] The regulation of cyanobacterial growth is also evidenced by the fact that mature leaf cavities contain large areas of space which are free of cyanobacteria. As in many other cyanobacterial symbioses, the vegetative cells of *Anabaena azollae* are somehow influenced to differentiate very high frequencies of heterocysts.

In the *Azolla-Anabaena* symbiosis, each shoot tip of an *Azolla* sporophyte is colonized by several filaments of *Anabaena azollae*. This colony, which comprises small, undifferentiated cyanobacterial filaments, serves as a persistent inoculum for developing leaf cavities. Cyanobacterial filaments are embedded in mucilage and protected from the environment by their proximity to the upcurved meristem, which is surrounded by newly differentiated leaf lobes.[6,7]

The ontogenic sequence of cavity inoculation and development has been described most

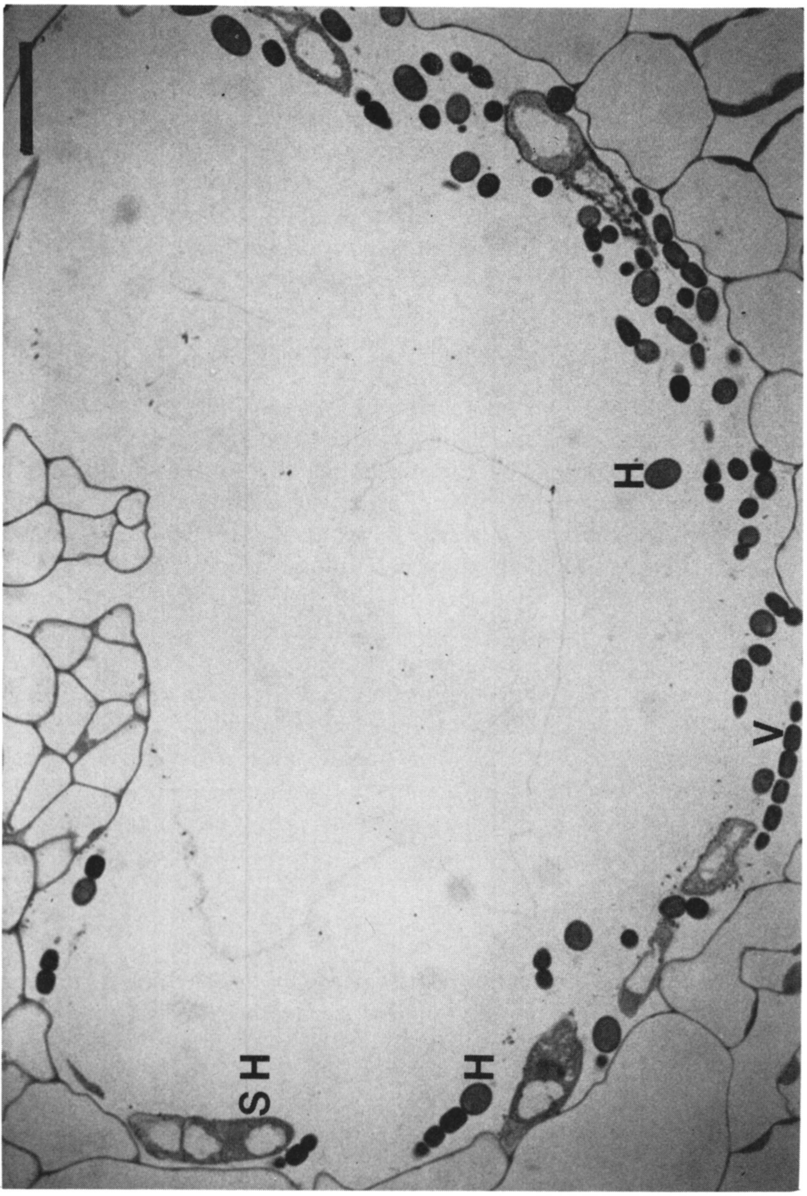


FIGURE 2. Light micrograph of an oblique longitudinal 1-μm-thick section through a mature *A. caroliniana* leaf cavity. Heterocysts (H) and vegetative cells (V) of *Anabaena azollae* line the cavity periphery. A simple hair (SH) is also apparent. Samples were fixed overnight at 4°C, poststained with OsO_4, and embedded in Spurr's resin. This section was stained with toluidine blue.[124] Bar = 25 μm.

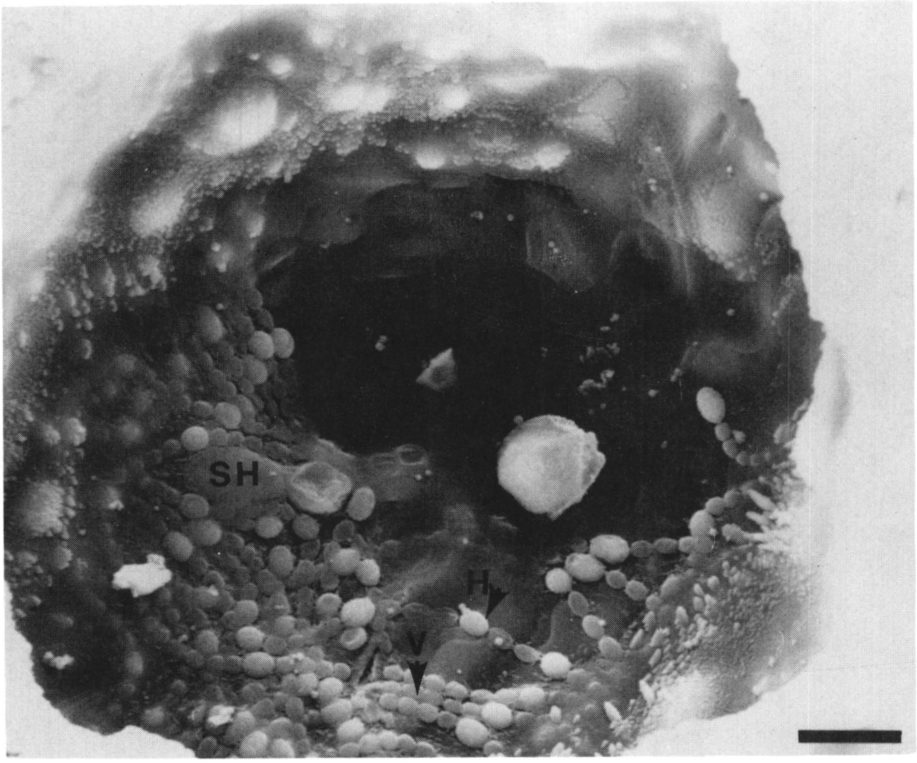


FIGURE 3. An SEM of an *A. filiculoides* leaf cavity, giving a three-dimensional visualization of the leaf cavity interior. Abbreviations: (V) vegetative cells, (H) heterocysts, and (SH) simple hair. Bar = 20 μm. (Reproduced with permission from Robins, R. J., Hall, D. O., Shi, D.-J., Turner, R. J., and Rhodes, M. J. C., *FEMS Microbiol. Lett.*, 34, 155, 1986.)

thoroughly for the *A. caroliniana* symbiosis.[19-22] At the apical meristem of an *Azolla* sporophyte, the axils of leaf primordia can rapidly differentiate specialized epidermal trichomes called primary branched hairs (PBH). These PBH, which consist of a stalk cell and three to five terminal cells, grow towards the apical *Anabaena* colony. The PBH of the three or four youngest leaves protrudes at the same time into the colony.[22] Eventually, a number of *Anabaena* filaments become entangled around the terminal cells of the PBH.

A transmission electron micrograph (TEM) of a vertical longitudinal section through a shoot tip of *A. caroliniana* is shown in Figure 4A. Several *Anabaena* filaments can be seen associated with the PBH of the third leaf primordium. A young leaf primordium that has recently initiated formation of a PBH is also visible. Figure 4B (inset) is a light micrograph of a thick section that was serially located to the thin section shown in Figure 4A. This micrograph shows the relative positions of the PBH, leaf primordia, and cyanobacterial filaments. Note the small, uniform size of the *Anabaena* filaments and the large PBH in close proximity to a leaf that has initiated cavity formation.

As the plant grows, the eldest PBH and its associated *Anabaena* filaments are displaced from the apical meristem to the proximal end of a depression that forms on the ventral side of the dorsal leaf lobe.[22] The cells surrounding this depression become meristematic and produce epidermal cells that grow inward, enclosing the PBH and *Anabaena* filaments. A pore remains at the top of the cavity where the meristematic epidermal cells meet.[1] By the time cavity formation is complete, the PBH has differentiated two or three body cells between (or to the side of) the stalk and terminal cells.[20]

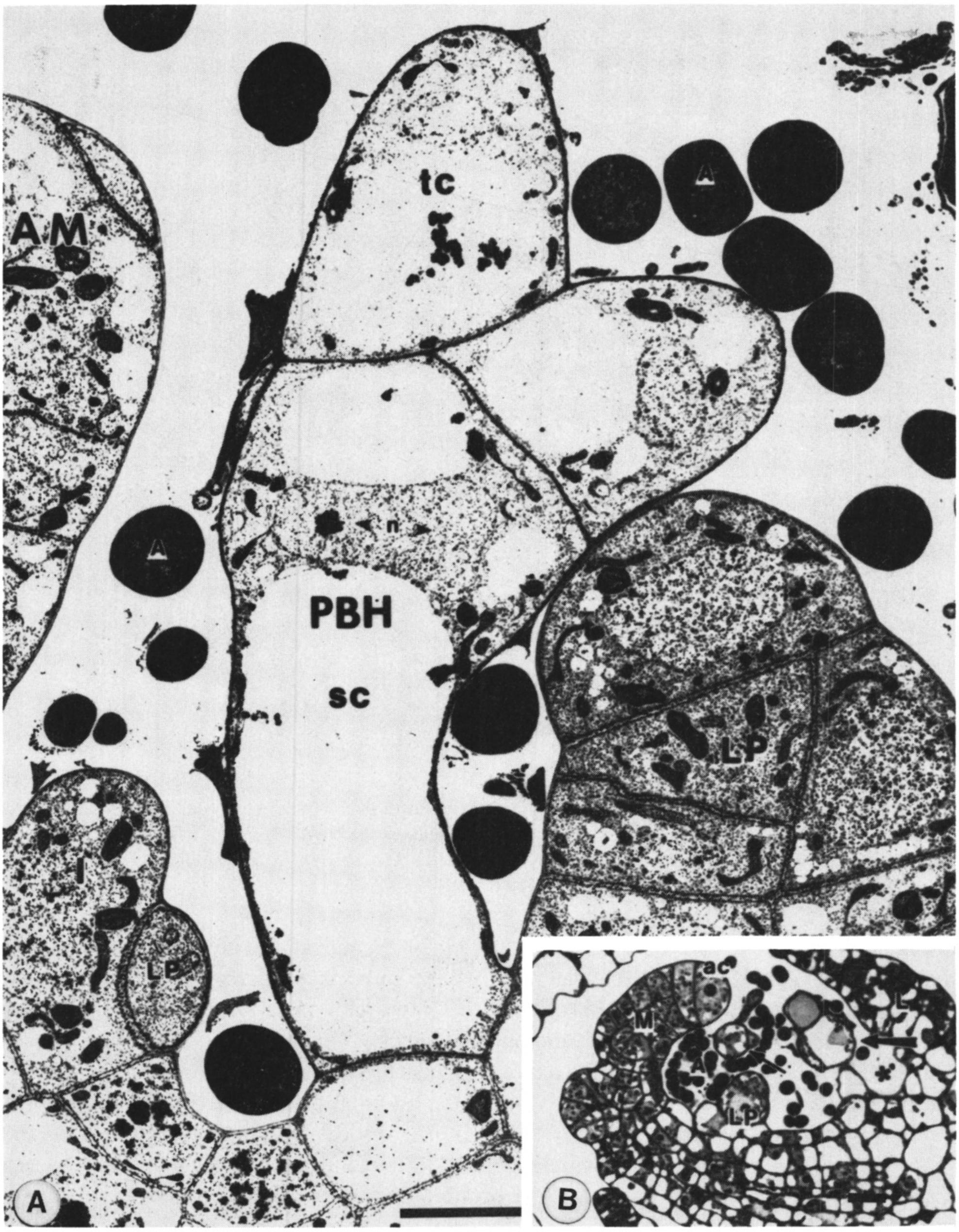


FIGURE 4. Micrographs of serial sections through an *A. caroliniana* shoot tip. (A) TEM of a vertical longitudinal section showing vegetative *Anabaena* cells (labeled A) surrounding the centrally located primary branched hair (PBH) near the apical meristem (AM). Branched hairs are intimately involved in the inoculation of developing *Azolla* leaf cavities with cyanobacteria. Only two of the three terminal cells (tc) of the PBH are visible in this section. A long stalk cell (sc) is intercalary to the terminal cells and cavity wall. As the PBH matures, body cells will develop between the stalk and terminal cells. A hair initial (I) associated with the youngest leaf primordium (LP) can be seen to the left of the large PBH. Bar = 2 μm. (B) Light micrograph of a thick section (1 μm) serially located to the thin section presented in Figure 4A, showing the proximity of the *Anabaena* colony (labeled A) to the leaf primordia (LP) and apical meristem (M), which is capped by the apical cell (ac). The locations of two PBHs are designated by arrows. Before *Anabaena* filaments are enclosed within a leaf cavity, the generative algal colony is protected from the environment by its proximity to the apical meristem and overarching young leaves (L). A developing cavity is seen to the right (★). Bar = 1 μm. (From Calvert, H. E., Pence, M. K., and Peters, G. A., *Protoplasma*, 129, 10, 1985. With permission of Springer-Verlag, New York.)

Hairs of two distinct morphological types become evident before cavity formation is completed. One type, termed a secondary branched hair, is similar to the PBH, except that it has only one or two body cells and two or three terminal cells. Each leaf cavity contains one primary and one secondary branched hair that are located at specific sites within the cavity.[22] These sites are close to, but separated from, the leaf trace by two or three layers of cells.[11,21] The other type of hair, called a simple hair, consists of a single stalk cell and a terminal cell. A TEM of a longitudinal section through the terminal cell of a simple hair is shown in Figure 5. Numerous *Anabaena* filaments and bacteria are often observed in close proximity to the hairs.

Whereas branched hairs are associated with a cavity throughout its development, simple hairs increase in number (up to 25) as a cavity matures.[19] Simple hairs apparently differentiate at random within a cavity, except in the lower distal quadrant.[20]

Ultrastructural Ontogeny of Cavity Hairs

The *Azolla* leaf cavity has no direct connection with the vascular system of the plant. Therefore, it has been suggested that cavity hairs may play an integral role in the transfer of nutrients between *Azolla* and its endosymbiont.[7,11,21] The ultrastructure of hair cells has been cited as evidence in favor of this hypothesis.

In a number of systems, transfer cells have been implicated to be involved in the transport of solutes over short distances.[23,24] Ultrastructurally, these cells are described as having a dense, organelle-rich cytoplasm that contains abundant rough endoplasmic reticulum and numerous wall ingrowths.[11] These wall protuberances are suggested to be the actual site of nutrient exchange.[24]

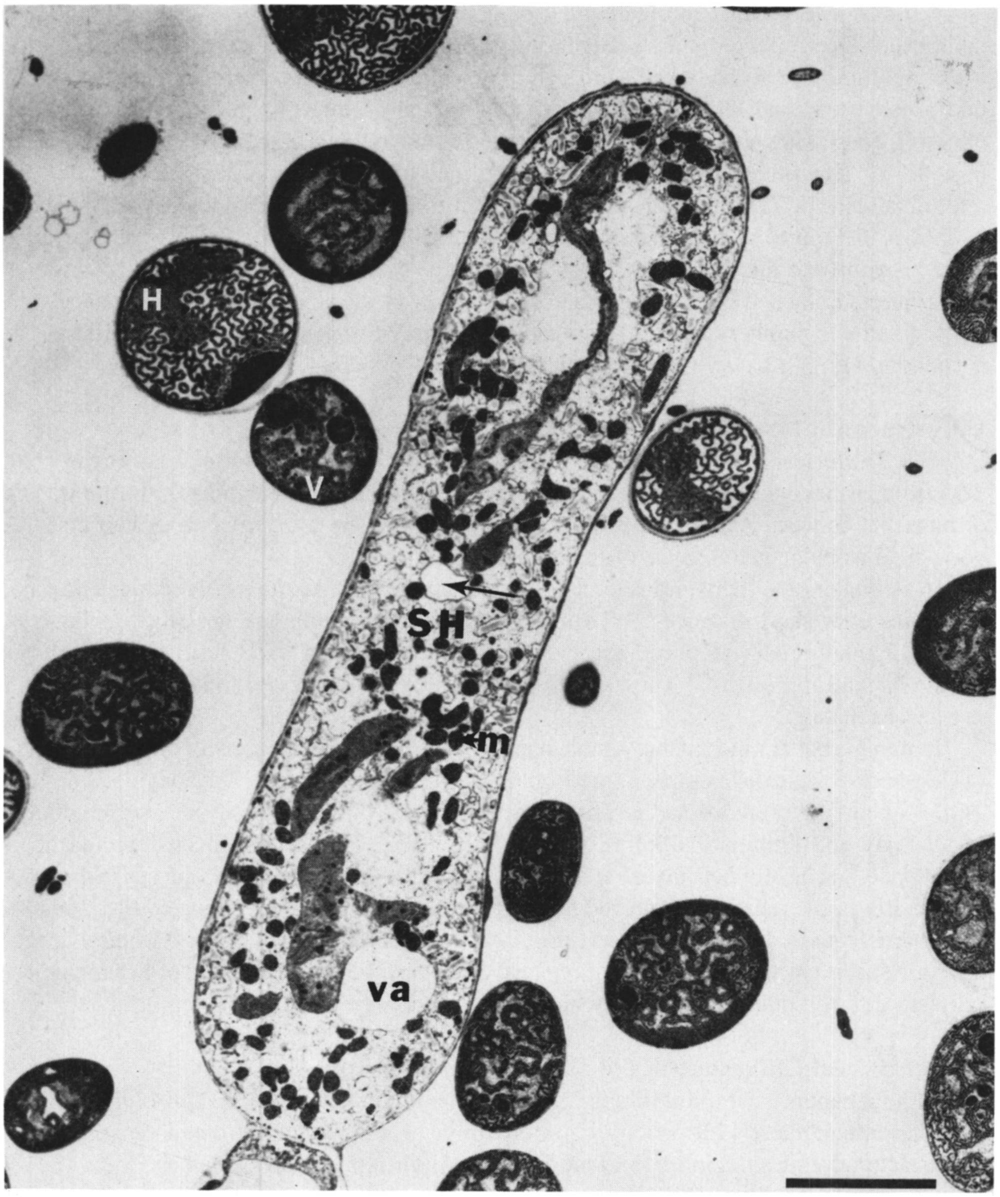
In *Azolla*, both branched and simple hairs exhibit characteristic transfer cell ultrastructure (TCU) during their development (see Figures 4A and 5). Terminal and body cells develop TCU soon after they are formed.[21] Stalk cells, however, exhibit TCU only after differentiation of all body and terminal cells has been completed.[21] TCU has also been reported in the foliar trace and in the cell layers intervening between the foliar trace and the leaf cavity, particularly in the region of branched hair attachment.[20] It has been postulated that branched hairs participate in the exchange of nitrogen between *Azolla* and *Anabaena*, whereas simple hairs are specifically involved in the exchange of carbon.[21] For a discussion of this hypothesis in relation to the ontogeny of cavity hairs, the reader is referred to Calvert et al.[21]

Ultrastructural Characteristics of Symbiotic *Anabaena azollae*

Although numerous physiological and biochemical studies on *Azolla-Anabaena* have been described, there is a paucity of information on ultrastructural characteristics of the cyanobacterial endosymbiont. Four studies have been reported to date, describing the endosymbionts of *A. filiculoides*,[25,26] *A. pinnata*,[27] and *A. caroliniana*.[28] Unfortunately, the growth conditions and fixation procedures used in these studies varied considerably, making it difficult (if not impossible) to compare the endosymbionts on an ultrastructural level. In addition, only one study has compared the ultrastructural features of the cyanobacteria present in cavities of different ages, and that paper did not include any transmission electron micrographs.[26] Thus, while general information on the ultrastructural characteristics of symbiotic *Anabaena azollae* exists, detailed studies will be required before accurate comparisons of different endosymbionts can be made. The following section is a compilation of the information currently available about the ultrastructural characteristics of *Anabaena azollae*. Some of our unpublished observations on the endosymbiont of *A. caroliniana* are also included.

Vegetative Cells

Anabaena filaments associated with the stem apex of *Azolla* are actively dividing, undifferentiated, and lack nitrogenase activity.[7,26,29] These vegetative cells are uniform in



IGURE 5. Terminal cell of a simple hair (SH) of *A. caroliniana*. This cell exhibits ultrastructural characteristics of transfer cells, including numerous mitochondria (m) and an invaginated cell membrane. Vacuoles (va) contain some granular material, except the smaller, centrally-located vacuole which is electron-transparent (arrow). Heterocysts (H) and vegetative cells (V) of *Anabaena azollae* are seen in close proximity to the hair, which has been suggested to function in the transport of carbon between the symbiotic partners. Bar = 5 μm. (From Peters, G. A. and Calvert, H. E., in *Algal Symbiosis*, Goff, L. J., Ed., Cambridge University Press, New York, 1983, 109. Copyright © 1983 Cambridge University Press. Reprinted with permission.)

size and usually contain numerous carboxysomes[26] (polyhedral bodies). The thylakoid membranes within these cells are often observed running parallel to the outer membrane.[26-28] Like free-living cyanobacteria, the vegetative cells exhibit a bilayered cell wall that is typical of Gram-negative bacteria.[27] Vegetative cells of this type from the endosymbiont of *A. caroliniana* are shown in longitudinal and cross sections in Figure 6. Polymorphic electron-

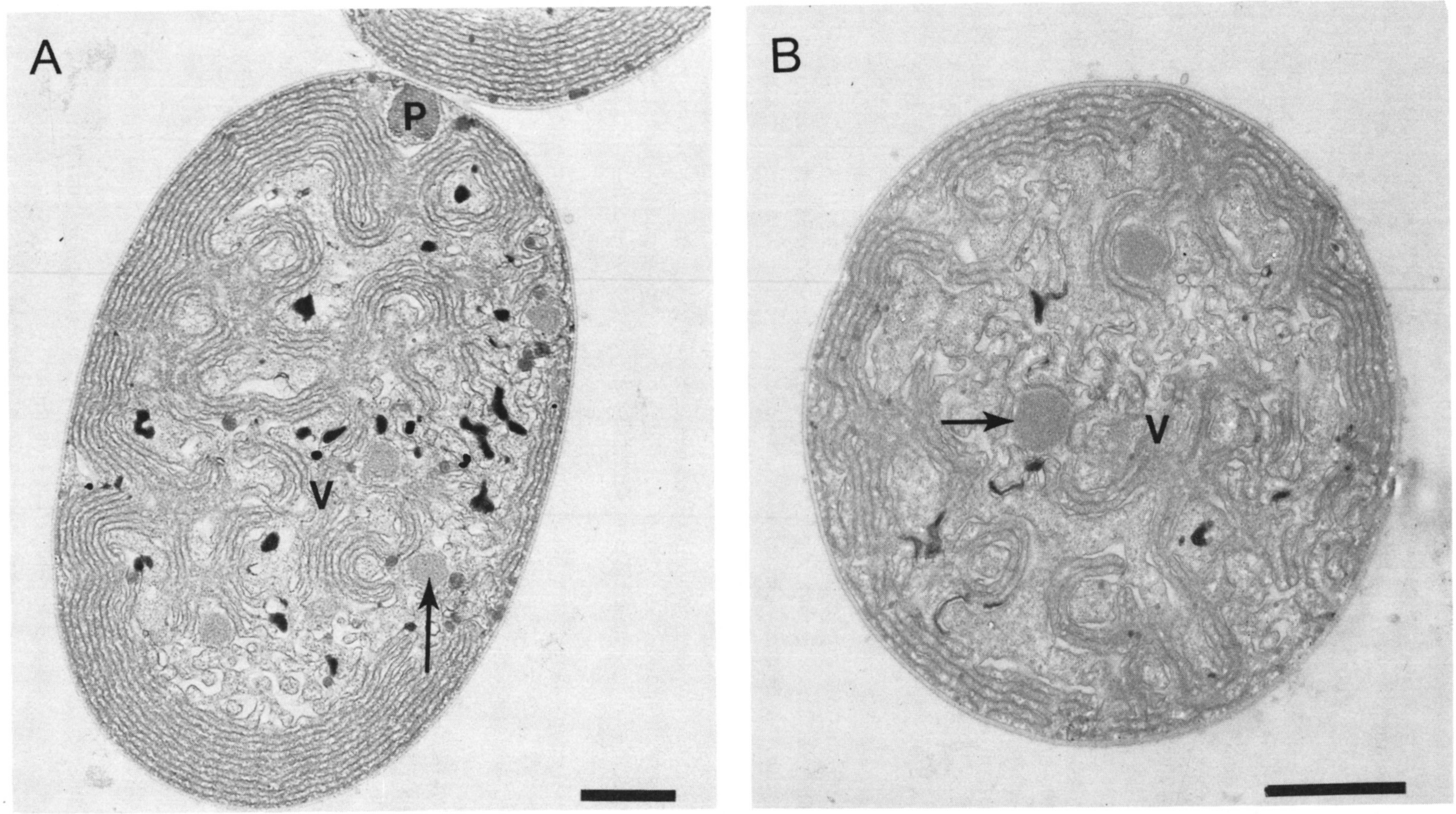


FIGURE 6. TEMs of (A) longitudinal and (B) cross sections of vegetative cells of *A. azollae* found within a cavity of *A. caroliniana*. Samples were prepared as described in Figure 2. Thin sections in this and succeeding figures were poststained with uranyl acetate and lead citrate. Within young vegetative cells (V), thylakoid membranes are generally arranged parallel to the cell wall, sometimes forming whirls. A plug (P) similar in appearance to the polar plugs of heterocysts in located between two adjacent vegetative cells. Polymorphic, electron-dense material frequently appears in vegetative cells and heterocysts. Numerous carboxysomes (arrow) are apparent, which disappear gradually during heterocyst differentiation. Bar = 1 μm in both (A) and (B).

dense material was frequently seen, in addition to carboxysomes, in the endosymbiont of *A. caroliniana*.

As leaf cavity closure commences, vegetative cells become enlarged and divide more slowly, and many of them differentiate into heterocysts.[26,29] This increase in vegetative cell size continues even after the onset of heterocyst differentiation.[26,29] In one report,[26] it was estimated that a vegetative cell increases approximately sevenfold in volume by the time it matures.

Mature cavities contain a heterogeneous population of cyanobacterial filaments. Within a filament, the relative shape and size of the vegetative cells is consistent. Between filaments, however, cell size and shape are extremely variable. In general, cells in the older leaf cavities contain fewer carboxysomes but more cyanophycin granules than do cells located in younger cavities.[26,27] In contrast to the apically located vegetative cyanobacterial cells that have thylakoids running parallel to the outer cell membrane, the thylakoid membranes of vegetative cells found within mature cavities are distributed throughout the cell cytoplasm.[26,27] These thylakoids have frequently been observed to form whorls or lattice structures, especially near the formation of new cross-walls.[27] In one study, granular, electron-transparent areas were observed between the thylakoid membranes of mature, dividing cells. These were assumed to be carbohydrate reserves (polyglucoside granules) that had been removed during the fixation process.[27] It was also reported that mature cells appeared to lack phycobilisomes.[27]

Heterocysts

The process of heterocyst differentiation from vegetative cells is manifested by several ultrastructural changes. These include the formation of extra envelope layers; a narrowing between the junctions of heterocysts and adjacent vegetative cells (formation of a neck); and the formation of large, electron-dense polar plugs (presumably cyanophycin granules) within the necks of heterocysts.[25,27] *Anabaena azollae* is unique in that polar granules are also frequently observed between adjacent vegetative cells.[27] The significance of these polar granules is currently unknown, but immunoelectron microscopy studies with antibodies against the Fe-protein of nitrogenase have indicated that these vegetative cells do not contain nitrogenase.

The first observable changes that occur during heterocyst differentiation in *Anabaena* are external. The cell becomes enlarged, and a loosely organized layer of envelope material appears outside of the cell wall.[25] As heterocyst development continues, the carboxysomes and cyanophycin granules gradually disappear. Membranes appear more diffuse in the center of cells and often form honeycomb lattices adjacent to the polar plugs. In addition to the cyanophycin plugs, mature heterocysts of *A. caroliniana* contain small, spherical granules of varying electron density (Figures 7A, C, and D). These have also been observed in heterocysts formed by *Mastigocladus laminosus*.[30] It is not yet clear if these are the same electron-dense inclusions that are observed in vegetative cells (Figure 6). Within *A. caroliniana* fronds, the ultrastructure of mature heterocysts is consistent, except with respect to the degree of membrane vesiculation. Our data suggest that membrane vesiculation in heterocysts increases with age. Heterocysts of various ages from the endosymbiont of *A. caroliniana* are shown in Figure 7.

Heterocyst frequency increases with leaf age in *Anabaena azollae*. The synchronous development of the fern and cyanobacteria was first noted by Hill[26,29] for *A. filiculoides*, and has since been substantiated for *A. caroliniana*,[31,32] *A. pinnata*,[32,33] and *A. mexicana*.[33] The number of heterocysts formed varies to some extent between species and with varying growth conditions,[33] but frequencies may reach as high as 30% in mature cavities.[29] In contrast, free-living *Anabaena* species exhibit uniformly spaced heterocysts that may reach frequencies of 10% when grown in the absence of a combined nitrogen source.[34]

Akinetes

Vegetative cells of some strains of *Anabaena azollae* may also form akinetes, or resting cells. The number of akinetes formed apparently varies among different *Azolla* species and with varying growth conditions. Whereas akinetes comprise a significant proportion (17%) of the cyanobacterial population in *A. caroliniana*,[35] they have not been observed in either *A. filiculoides*[26] or *A. pinnata*.[27] The ultrastructural characteristics that distinguish akinetes from vegetative cells in a number of free-living cyanobacterial species have been described.[36-39] These studies indicate that characteristic features of akinetes include a thick, multilayered external envelope and large amounts of cyanophycin. Ultrastructural studies on akinetes of the *A. caroliniana* endosymbiont were recently initiated in this laboratory; cross and longitudinal sections through such akinetes are shown in Figure 8. It has also been reported that akinetes are considerably larger than vegetative cells.[37] The relative sizes of akinetes and heterocysts are seen clearly in Figure 8C.

The function of akinetes in *Azolla* leaf cavities is unknown. However, cyanobacterial cells resembling akinetes constitute the cyanobacterial inoculum in sporocarps of at least some *Azolla* species (see Subsection "Sporulation" in Section "Reproduction").

ENDOSYMBIOTIC BACTERIA

The mutualistic association between cyanobacteria and bacteria in natural waters has been well documented.[40-42] Radiotracer techniques have been used to show the direct transfer of organic substrates produced by algae during photosynthesis to the associated microflora.[42,43] In the *Azolla-Anabaena* symbiosis, transfer of nutrients between the plant and its endosymbiont occurs within the confines of the dorsal leaf cavity.[44,45] Given this constant supply of organic nutrients, in addition to those provided through the senescence of cyanobacterial cells, it is not surprising that bacteria are invariably found within *Azolla* cavities.[7,46,47]

ULTRASTRUCTURE OF BACTERIA WITHIN *AZOLLA* LEAF CAVITIES

The presence of bacteria within *Azolla* leaf cavities can be noted in transmission electron micrographs from ultrastructural studies.[7,11,21,22] Results from such studies in our laboratory show that the bacteria associated with *A. caroliniana* often contain electron-dense inclusions within their cytoplasm (Figure 9). These bacteria lack the thick peptidoglycan layer which is typical of Gram-positive organisms.[48] Numerous morphologically diverse bacterial cells have been observed within lysed cyanobacterial cells (Figure 9C). The large number of cyanophycin granules and the extra cell wall layers suggest that the cell shown in Figure 9C was probably an akinete, but bacteria have also been seen feasting on vegetative cells.

The presence of bacteria within cavities of endophyte-free *Azolla* fronds has also been noted.[46] Therefore, even in the absence of the cyanobacterial endophyte, the nutrient levels within leaf cavities are sufficient to support bacterial growth.

CHARACTERIZATION AND ENUMERATION OF BACTERIAL ISOLATES

To date, endosymbiotic bacteria have been isolated and characterized from four *Azolla* species.[48] These isolates were obtained from homogenates of surface-sterilized *Azolla* fronds. Dilutions of fern extracts were plated onto plate count agar and incubated under aerobic conditions. The results of these studies indicated that, regardless of the *Azolla* species used as an inoculum, *Arthrobacter* spp. comprised approximately 90% of the bacterial colonies that appeared under these growth conditions.[48,49] The results of physiological and nutritional studies have indicated that these bacterial isolates are most similar to *Arthrobacter globiformis* Conn and Dimmick.[48] Earlier studies indicated that bacterial isolates from *Azolla* were aerobic organisms, incapable of nitrogen fixation.[7,46,47,50] In agreement with this, bacterial growth

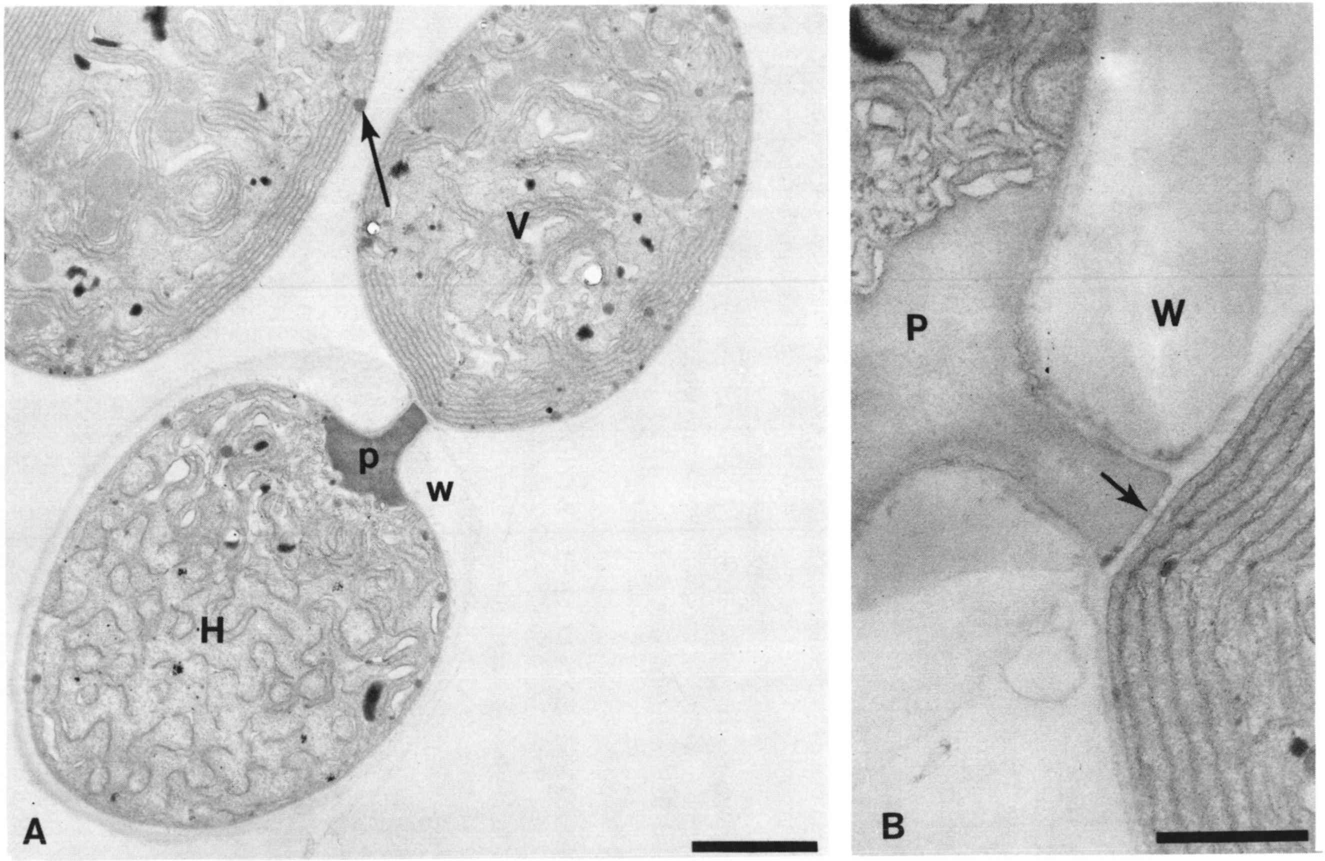


FIGURE 7. TEMs of heterocysts (H) and adjacent vegetative cells (V) in *Anabaena azollae* associated with *A. caroliniana*. (A), (C), and (D) illustrate the characteristic features of heterocyst ultrastructure, including a thick cell wall (W), prominent polar plugs (p), and a narrow neck at the junction of heterocysts and vegetative cells. Spherical, electron-dense particles of unknown nature are frequently observed (arrow). A typical honeycomb configuration of membranes adjacent to polar plugs is evident in (C) and (D). The degree of membrane vesiculation appears to increase with heterocyst maturity; compare (A), (C), and (D). Bar = 1 µm in (A), (C), and (D). (B) is a high-magnification micrograph of the neck region of the heterocyst presented in (A). Microplasmodesmata (arrow) between the two cells are clearly visible. Bar = 0.5 µm in (B).

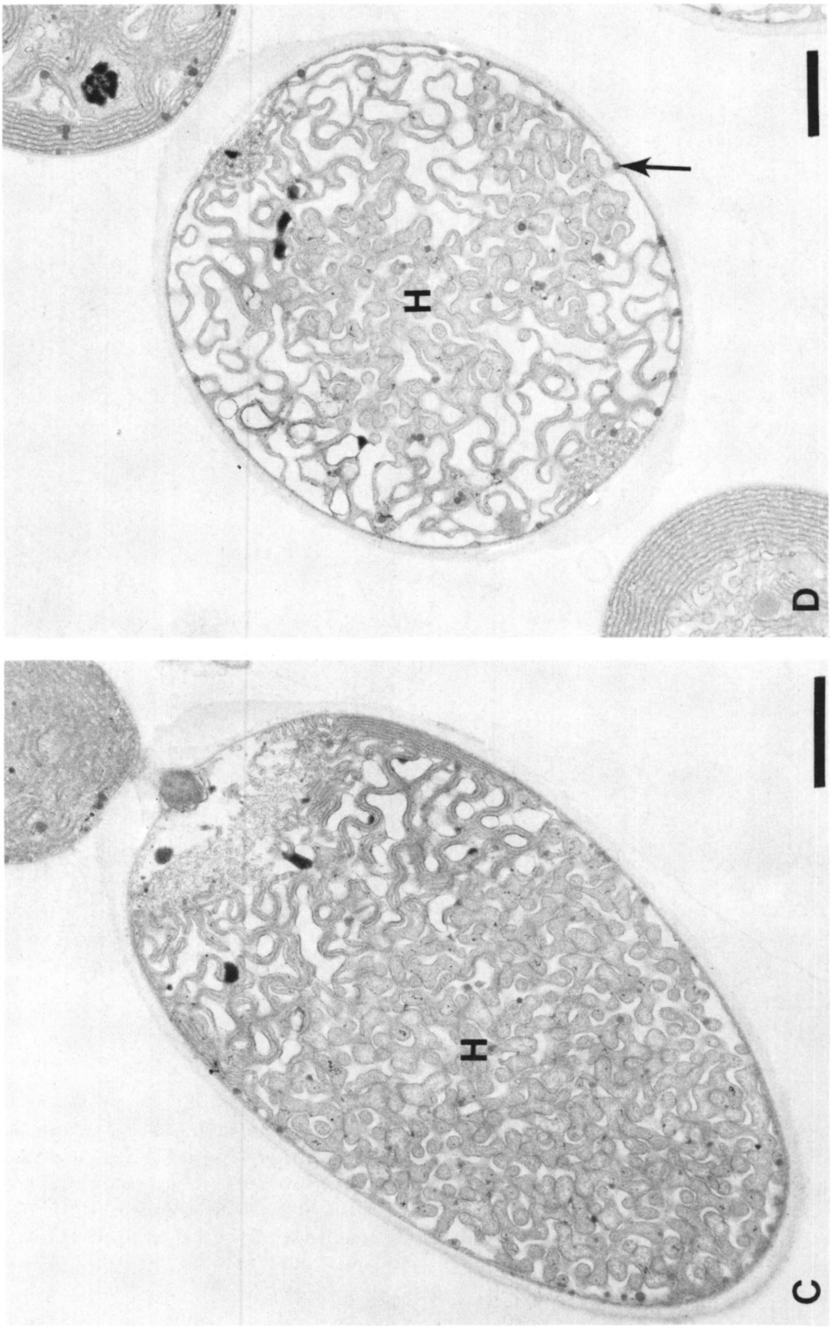


FIGURE 7 (continued).

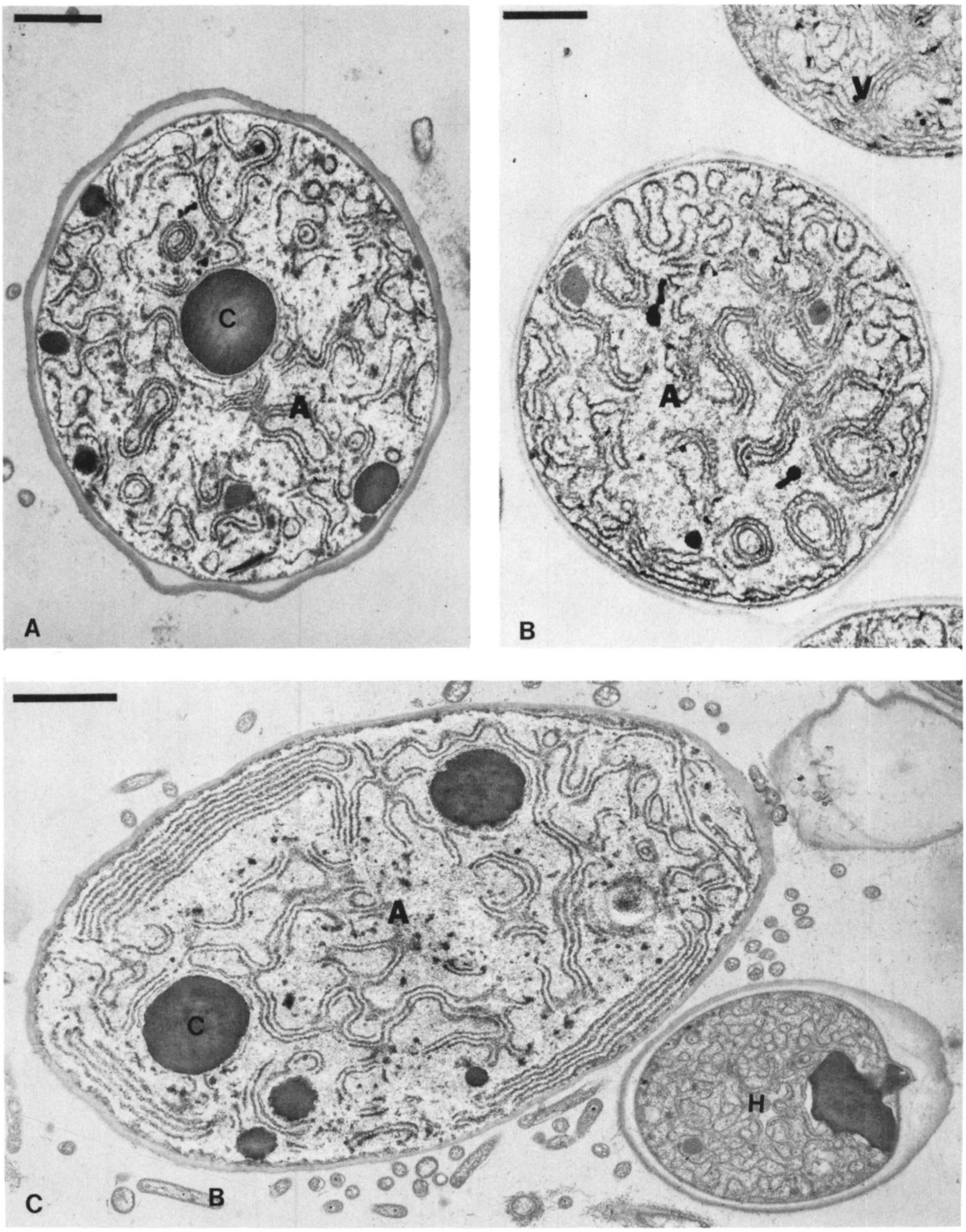


FIGURE 8. TEMs of cross (A), (B), and oblique (C) sections of akinetes in *A. caroliniana* leaf cavities, illustrating the ultrastructural characteristics of these cyanobacterial resting cells. The extra wall layer of akinetes is evident in (B) through comparison of the akinete (labeled A) wall with that of the vegetative cell (V). Characteristic features also include membrane rearrangements and the presence of numerous cyanophycin granules (labeled C). In (C), the relative sizes of the akinete (labeled A), heterocyst (H), and bacteria (labeled B) should be noted. Bar = 1 μm in (A), (B), and (C).

was not observed in fern extracts which had been incubated anaerobically on thioglycollate agar.[18]

A direct linear relationship was shown to exist between leaf age and the number of bacteria isolated from *A. filiculoides*, *A. caroliniana*, and *A. mexicana*.[49] However, this was not true in *A. pinnata*, which contained very low numbers of bacteria. The highest bacterial

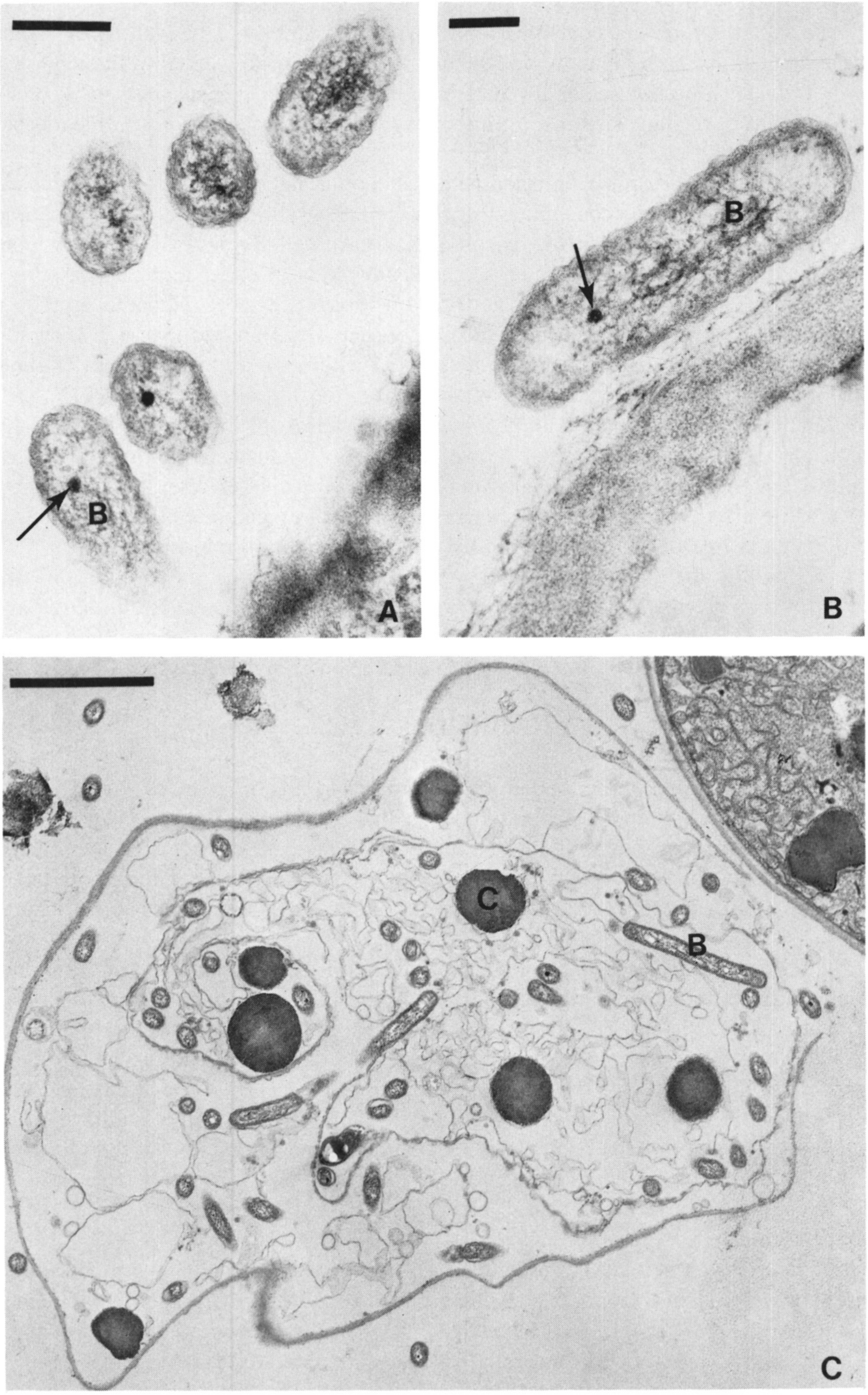


FIGURE 9. TEMs of endosymbiotic bacteria within *A. caroliniana* leaf cavities. Electron-dense granules (arrow) can be noted in both oblique (A) and longitudinal (B) sections. The lack of a thick peptidoglycan layer typical of gram-positive cells should be noted. In (C), numerous bacteria are observed within a senescent cyanobacterial cell, probably an akinete. Bar = 0.1 μm in both (A) and (B), bar = 1 μm in (C).

numbers (roughly 3 × 10^5 bacteria per 15th leaf) were isolated from *A. filiculoides; A. mexicana* and *A. caroliniana* contained somewhat lower numbers.[49] It has also been estimated that the numbers of bacteria and cyanobacteria are approximately equal in the tenth leaf of *A. caroliniana*.[18] Because bacterial populations within *Azolla* species were stable and predictable, it has been suggested that a tripartite symbiosis between the plant, cyanobacteria, and bacteria may exist.[49]

It is probable that *Azolla* is infected with bacteria throughout its life cycle. The bacteria are most likely transmitted concomitantly with the endosymbiont during asexual reproduction. However, it is unclear whether bacteria are transmitted during sexual reproduction, an issue that could be addressed by microscopic examination of either fertile megasporocarps or leaf cavities of *Azolla* sporophytes generated from surface-sterilized sporocarps.

Petro and Gates[49] estimated that glucose concentrations greater than 5 m*M* would be required to support the bacterial populations found within *Azolla* leaf cavities. The metabolism of 5 m*M* glucose would result in the production of at least 30 m*M* CO_2 and the reduction of at least 30 m*M* O_2.[49] In other systems, bacterial utilization of O_2 released during cyanobacterial metabolism has been shown to stimulate cyanobacterial nitrogen fixation.[41] It has also been suggested that algal growth is stimulated by the CO_2 released during bacterial heterotrophic activity.[51-53] The development of bacteria-free cultures of *Azolla* would help to elucidate the role of these bacteria in the *Azolla-Anabaena* symbiosis.

In addition to the aforementioned endophytic microorganisms, an abundant microflora is normally associated with the roots and leaves of *Azolla* sporophytes. It is usually possible to remove these epiphytes by surface-sterilizing plants with hypochlorite. We have found that this method is especially effective when accompanied by brief periods of sonication.

REPRODUCTION

Azolla can reproduce either sexually via sporocarp formation or asexually via vegetative fragmentation. Most strains of *Azolla* reproduce almost exclusively via vegetative fragmentation.[54] In this process, abscission layers that form at the base of lateral branches facilitate the separation of branches from the main rhizome.[55] The vegetative mode of reproduction is quite rapid in *Azolla*, with a doubling time of approximately 2 d under optimal laboratory conditions.[5]

The less frequently observed type of reproduction is sexual reproduction via sporocarp formation. Sporulation occurs regularly in a few strains of *Azolla*, but only rarely in others.[56] The precise factors that induce sporulation have not been elucidated, but sporocarp formation frequently follows exposure to adverse conditions.[1] The factors currently thought to be most important are light intensity,[54,57,58] temperature,[54,58] plant density.[54,58] and photoperiod.[59,60]

Numerous studies have described the ultrastructural manifestations of the sporulation process; for a recent review, see Perkins et al.[61] A schematic diagram of the reproductive cycles of *Azolla* is presented in Figure 10.

SPORULATION

Azolla is a heterosporous fern which produces two types of sporocarps on the same plant. Sporocarps form at lateral branch points on the ventral side of the sporophyte, replacing the ventral lobe of the first leaf on a branch.[62,63] During this process, the partner dorsal lobe retains its normal structure and function.[1,63] Sporocarps are attached to the plants by short stalks and, prior to maturation, are enclosed by a common, protective single-celled layer called the involucre[63] or hood.[62] This protective covering originates from the dorsal leaf lobe.[55,63] Sporocarps occur in pairs in all *Azolla* species except *A. nilotica*, in which they occur as tetrads.[4,64] A pair of megasporocarps partially covered by a hood is shown in Figure 11A. Sporocarp pairs may consist of two large globular microsporocarps (male), two small

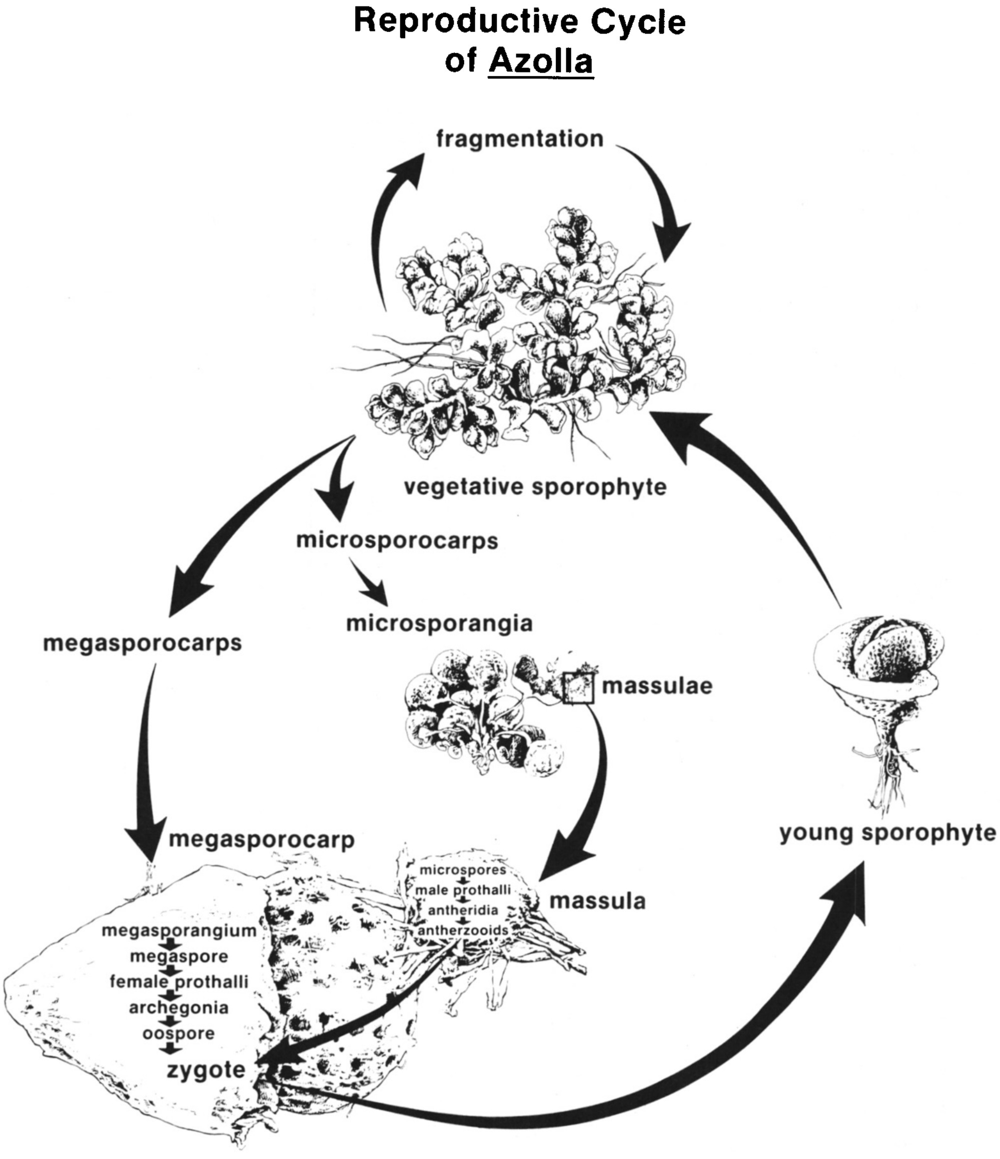

Reproductive Cycle
of Azolla
fragmentation
vegetative sporophyte
microsporocarps
microsporangia
megasporocarps
massulae
megasporocarp
microspores
male prothalli
antheridia
antherozoids
massula
megasporangium
megaspore
female prothalli
archegonia
oospore
zygote
young sporophyte

FIGURE 10. Schematic drawing of the reproductive cycle of *Azolla*.

ovoid megasporocarps (female), or one of each.[63,65] The ratio of megasporocarps to microsporocarps is influenced by environmental conditions.[1] The two morphologically distinct sporocarp types that occur in *A. mexicana* Presl are shown in Figure 11B.

Structure of Megasporocarps

Each megasporocarp contains one megasporangium (consisting of a single megaspore and the megaspore apparatus) and a distally located colony of *Anabaena*. Before maturation, the megasporocarp is enclosed by a two-layered indusium, except at the distal end, where an indusial pore is located.[63,65] Following maturation and the release of megaspores into the water column, the proximal portion of the indusium decays, while the distal portion hardens due to lignification and the deposition of tannin.[63] The distal portion remains associated with the megaspore apparatus until it is displaced by the emergence of the sporophyte embryo.[63,65]

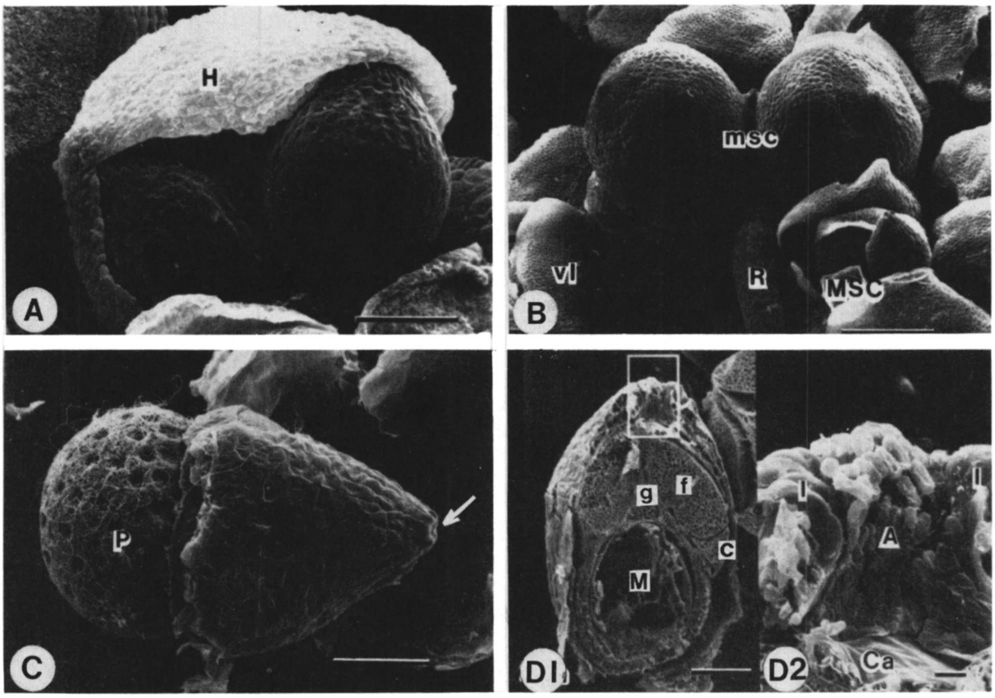


FIGURE 11. SEMs of *A. mexicana* megasporocarps. (A) Pair of megasporocarps with the protective hood (H) partially removed. Bar = 200 μm. (B) Two sporocarp pairs, showing the relative sizes of the large, globular microsporocarps (msc) and the smaller, ovoid megasporocarps (MSC). Roots (R) and sporocarps form on the ventral surface of the sporophyte at the point of lateral branch attachment to the main rhizome. A ventral leaf lobe (vl) on a lateral branch is also visible. Bar = 500 μm. (C) A megasporocarp with the proximal portion of the indusium manually removed to reveal the pitted perine (P) covering of the megaspore. Long thin filamentous processes emanate from the perine surface. The indusial pore (arrow) is distally located. Bar = 100 μm. (D1) A bisected megasporocarp, showing the relative positions of the apical cap (within the box), floats (f), collar (c), gula (g), and megaspore (M). Bar = 100 μm. (D2) A magnification of the distal portion of the megasporocarp, showing the location of the *Anabaena* cells (labeled A) between the indusium (I) and apical cap (Ca). Bar = 10 μm. (From Calvert, H. E., Perkins, S. K., and Peters, G. A., *Scanning Electron Microsc.*, 3, 1499, 1983. With permission.)

One of the primary functions of this hardened indusium is to protect the *Anabaena* colony (see below).

A megasporocarp from which the proximal portion of the indusium has been manually removed, thereby revealing the perine (perispore covering), is illustrated in Figure 11C. The topography of this covering is distinct for each *Azolla* species.[61] Hair-like filaments, referred to as filosum[66] or the capture mechanism,[67] can be seen emanating from the perine surface. These filaments are found on the perine of all *Azolla* species except *A. nilotica*.[4]

A bisected megasporocarp from *A. mexicana* is shown in Figure 11D. The large, proximally located megaspore contains protein bodies, lipid globules, amyloplasts, and polysaccharide granules, and reportedly functions as a food reserve.[67] Above the megaspore, the gula extends underneath and between the floats. A collar (girdle) extends around the circumference of the megasporangium between the perine and the floats. The number of floats differs between the two *Azolla* sections (see later). The apical cap lies above the floats. Numerous *Anabaena* cells are located between the apical cap and the indusium (Figure 11D2). The structural integrity of the megaspore apparatus is maintained through an intermeshing of filaments that are attached to the floats, gula, and cap.[68] The development of the megaspore apparatus in *A. microphylla* has been described in detail by Herd et al.[69]

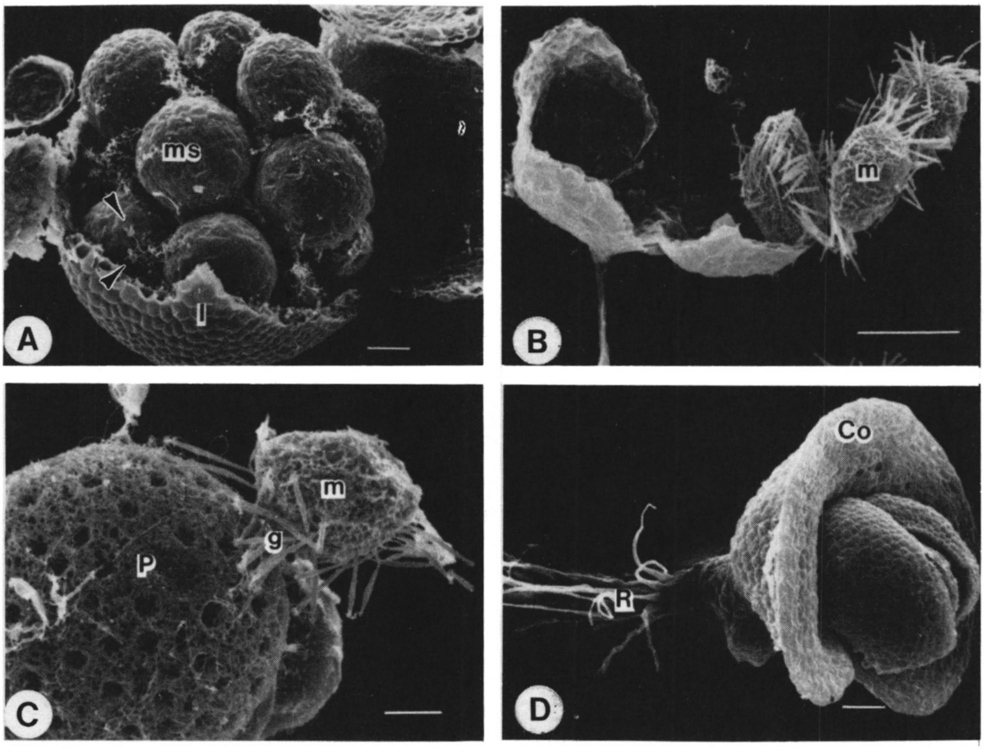


FIGURE 12. SEMs. (A) Numerous, spherical microsporangia (ms) within a microsporocarp of *A. mexicana*. The indusium (I) has been partially removed. Cyanobacterial cells (arrow) can be seen on the microsporangial surface. Bar = 100 μm. (B) A microsporangium which has been manually opened to release the massulae (m). Three of the four massulae are visible in this micrograph. Arrow-like projections (glochidia) cover the surface of each massula. Bar = 100 μm. (C) Attachment of a massula (m) to the perine (P) of an *A. mexicana* megasporocarp. The glochidia (g) become entangled in filaments emanating from the perine surface prior to fertilization. Bar = 50 μm. (D) An *A. mexicana* plantlet, with an embryonic leaf (Co) and primary root (R) after release from the megaspore apparatus. Bar = 100 μm. (From Calvert, H. E., Perkins, S. K., and Peters, G. A., *Scanning Electron Microsc.*, 3, 1499, 1983. With permission.)

All sporocarps develop as megasporocarps until the primary sporocyte has divided into 32 megaspore nuclei. If megaspore development proceeds, 31 of these nuclei degenerate and the surviving nucleus forms the megaspore. However, if all 32 nuclei abort and the megasporangium senesces, then microsporocarp development occurs.[63,65]

Structure of Microsporocarps

Azolla microsporocarps are globular structures that are significantly larger than megasporocarps (Figure 11B). Microsporocarps are surrounded by a membranous indusium that decays soon after maturation.[63] Removal of the indusium reveals the presence of between 8 and 130 spherical microsporangia[70] (Figure 12A). *Anabaena* cells have been observed on the surface of microsporangia,[65] but the significance of their presence is unknown. However, it is possible that the *Anabaena* cells are simply residual because each sporocarp begins development as a megasporocarp.

When released into the water column, the microsporangia are attached to a placentum by thin stalks.[63,65] Each fragile microsporangium contains between three and ten massulae[70] that are composed of hardened mucilage containing high concentrations of sporopollenin.[67] Encased within each massula are either 32 or 64 microspores.[61] Massulae of all extant *Azolla* species except *A. nilotica*[4] bear filaments known as glochidia, that facilitate attachment of the massula to the megaspore prior to fertilization. An *A. mexicana* microsporangium which has been opened to reveal the long, barbed glochidia on the surface of the massulae is shown in Figure 12B.

Resistance

Both mega- and microsporocarps have been shown to contain large amounts of sporopollenin, which makes them very resistant to UV irradiation, desiccation, nonoxidative chemical attack, and biodegradation.[71] This is evidenced by the abundance of megaspores found in the fossil record; to date, more than 40 fossil species of *Azolla* have been described.[72,73]

Fertilization and Development

Following maturation, sporocarps separate from the mother sporophyte and sink to the bottom of the water body. It is not clear whether a dormant period is required prior to fertilization[63] and germination. Germination, production of antheridia, and release of antherozoids occur while a microspore is still embedded within a massula. Within the megasporocarp, megaspores germinate into female gametophytes, each of which bears one or more archegonia containing a single egg cell (oocyte). Following the attachment of massulae to the megasporocarp (Figure 12C), antherozoids move through the gelatinized massulae to fertilize oospores within the archegonia.[70] Zygote formation occurs within the megaspore apparatus below the water surface.[63]

Germination

Identification of the factors influencing the germination rate of *Azolla* sporocarps is an area of current research interest. At present, those factors considered to be most important are light[54,56] and temperature.[56,60]

As an *Azolla* embryo grows, the hardened distal indusium of the megaspore apparatus hinges to one side and the plantlet is released. A recently germinated *A. mexicana* sporophyte (consisting of an embryonic leaf, root, and foot) is shown in Figure 12D. The plantlet floats to the water surface after one or two leaves have been formed[61,63] and initially grows in the vertical direction. In *A. filiculoides*, the plantlet floats horizontally on the water surface after four or five leaves have formed, and branching begins after the sixth leaf is visible.[74] Within 1 to 2 months, a young *Azolla* plantlet attains full fern size.[31,54]

Sexual Hybridization of *Azolla* Sporocarps

The formation and isolation of *Azolla* hybrids has been reported recently.[56] When megasporocarps of *A. filiculoides* were crossed with microsporocarps of *A. microphylla* (or vice versa), the germination of a few *Azolla* hybrids was observed. Initial studies indicated that these *Azolla* hybrids exhibit the increased resistance to higher temperatures that is characteristic of *A. microphylla*. Further characterization of these hybrids is currently in progress at the Fujian Academy of Agricultural Science, China, and at the International Rice Research Institute (IRRI), Los Baños, Philippines.

Attempts to hybridize *A. mexicana* or *A. pinnata* with other *Azolla* strains have been unsuccessful.[56] One possible explanation for the failure to establish an *A. pinnata* hybrid was that the massulae of this Old World species could not effectively attach to the megasporocarps of the New World species of *Azolla*. Therefore, it is likely that fertilization did not occur.

Maintenance of the Symbiosis

The symbiotic association between *Azolla* and *Anabaena* is maintained throughout both sexual and asexual modes of reproduction. Because vegetative sporophytes contain a generative *Anabaena* colony at each branch apex, each plant produced by fragmentation carries its own inoculum.

The source of inoculum and mechanism whereby *Anabaena* is inoculated into the megaspore during sporogenesis are not clear. It is known that, as the apex of the sporophyte emerges from the megaspore apparatus, it is inoculated with *Anabaena*. The nature of the cyanobacterial inoculum apparently differs among different *Azolla* species. In *A. mexicana*, resting cells that germinate into undifferentiated cyanobacterial filaments close to the time of female gametophyte development serve as the inoculum.[65] In *A. filiculoides*, however, the cyanobacterial inoculum reportedly consists of resting cells.[31] It was observed that these resting cells are approximately twice as large as cells growing at the shoot apex of *A. filiculoides*.[31] In regard to the mechanism of transfer, Calvert et al.[65] have suggested the involvement of unbranched trichomes found at the proximal end of megasporocarps in a process analogous to cavity infection.

Considerable growth of undifferentiated *Anabaena* filaments has been reported in developing megasporocarps of *A. filiculoides*.[31,58] The cyanobacterial filaments in mature megasporocarps lack heterocysts, they do not fix nitrogen, and their cells are apparently in a resting state. Several researchers have referred to these resting cyanobacterial cells as akinetes. However, Becking[31] has reported that these cells have a cell wall that is more similar to that of vegetative cells than that of akinetes.

Reinfection of Symbiont-Free *Azolla* Megasporocarps with *Anabaena azollae*

An artificial reconstruction of the *Azolla-Anabaena* symbiosis from heterologous megasporocarps was achieved recently.[75] It was reported that, if the apical cap and indusium were excised from a fertile megaspore apparatus, then the plants emerging from these megaspores were *Anabaena*-free. Furthermore, if an apical cap from a different fertile megasporocarp was placed on top of the excised megasporocarp, then reinfection with the cyanobacteria associated with the transplanted apical cap could be demonstrated.[75] This technique was successfully used to reinfect megaspores from *A. microphylla* with cyanobacteria of *A. filiculoides* and vice versa. Megaspores of an endophyte-free strain of *A. filiculoides* were also reinfected using this method. These reconstructions were verified using SEM, monoclonal antibodies, and nitrogen fixation assays. Attempts to reinfect excised megaspores with free-living strains of *Anabaena azollae* resulted in "incomplete symbioses".[75] In this case, some of the cyanobacterial cells infected the leaf cavity, but most of them were found on the outside of the frond. Low rates of acetylene reduction were observed in these incomplete symbioses. One of the factors that was reported to be crucial in the establishment of the symbiotic state was the synchronous growth of the two partners. Thus, although the fern appeared to be infected with the new cyanobacteria in many cases, the difference in growth rates eventually resulted in one partner outgrowing the other.[75]

As discussed in the following chapter, overwintering and oversummering of sporophytes are major problems in the practical use of *Azolla* as a biofertilizer. Because the factors that induce and control sporulation processes are still poorly understood, sporocarps are not yet being used on a widespread basis for the maintenance of strains and the inoculation of crops with *Azolla*. Nevertheless, the potential conservation of time, cost, and manpower that would be achieved through the use of sporocarps is recognized. Therefore, the regulation of sporocarp development and germination are presently areas of great research interest.[56,74,76]

TAXONOMY

AZOLLA

The taxonomy of *Azolla* has been revised frequently since the genus was first described by Lamarck in 1783.[77] Historically, *Azolla* has been placed in the family Salviniaceae with

TABLE 1
Classification of *Azolla*

Division:	Pteridophyta	
Class:	Filicopsida	
Order:	Salviniales	
Family:	Azollaceae	
Genus:	*Azolla*	
Sections:	*Azolla* (New World species)	*Rhizosperma* (Old World species)
Species:	*A. caroliniana*	*A. pinnata*
	A. filiculoides	*A. nilotica*
	A. mexicana	
	A. microphylla	
	A. rubra[a]	
Varieties:		*A. pinnata* var. *imbricata*
		A. pinnata var. *pinnata*

[a] Formerly classified as *A. filiculoides* var. *rubra*, *A. rubra* is currently recognized as a distinct species.[1,61,66] *A. rubra* is also frequently referred to as *A. japonicum*.[110]

Salvinia, another heterosporous water fern.[16] However, major differences between the two, particularly with respect to reproductive structures, have resulted in the recategorization of *Azolla* into the monotypic family Azollaceae.[1,16,63,66,70] The current classification scheme of the genus *Azolla* is presented in Table 1.

The seven extant species of *Azolla* are divided into two sections, *Azolla* and *Rhizosperma*. The primary basis for this division is sporocarp morphology. More specifically, species that belong to section *Azolla* exhibit three floats on the megaspore apparatus, whereas species in section *Rhizosperma* have nine.[16] In the latter case, the floats are arranged on the megaspore in two groups, with three above and six below. The two sections also differ with respect to the nature of the glochidia that are attached to the massulae. Long, barbed glochidia cover the surface of the massulae in members of section *Azolla*.[61] In contrast, the massulae of *A. nilotica* lack glochidia, and those of *A. pinnata* are irregular, unbarbed processes that may be either branched or unbranched.[63]

Identification at the species level is often difficult because it requires electron microscopic analysis of the reproductive structures. While sporocarp morphology has been very useful in the taxonomic classification of *Azolla*, it is limited by the fact that sporulation is unpredictable and infrequent in nature, so that specimens are often sterile.[54] Unfortunately, this problem has led to discrepancies in the classification of previously collected material.[3,78] In the absence of sporulating material, a tentative taxonomic key for *Azolla* species based on differences in sporophyte morphology has been presented.[1]

Section *Rhizosperma* comprises *A. nilotica* DeCaisne and *A. pinnata* R. Brown.[16] Based on differences in leaf branching morphology, *A. pinnata* is further divided into the varieties *A. pinnata* var. *pinnata* and *A. pinnata* var. *imbricata* (Roxburgh) Bonaparte.[79] Section *Azolla* comprises five species, which include *A. caroliniana* Willdenow, *A. mexicana* Presl, *A. microphylla* Kaulfuss, *A. filiculoides* Lamarck (type species), and *A. rubra* R. Brown.[1,61,66] In regions where *Azolla* is common, additional varieties are locally recognized.[1,58]

Members of section *Rhizosperma* are easily distinguished from each other and from members of section *Azolla* on the basis of vegetative morphology.[61] The sporophytes of both *A. pinnata* varieties exhibit a triangular shape, whereas *A. nilotica* is easily identified by its large size. Identification of *A. nilotica* is also facilitated by its limited geographic distribution[1] (see subsection on distribution).

In contrast to the differing sporophyte morphologies of *A. nilotica* and *A. pinnata*, members of section *Azolla* exhibit very similar vegetative characteristics. In addition, the vegetative morphology of these species can change considerably under different growth conditions.[1-3] Because of these factors, the precise identification of these species relies on the structural characteristics of the megasporocarps, rather than on sporophyte morphology.

The topography of the perine covering on the megaspore is most important in specific classification because it is unique to each species. Perkins et al.[61] have recently reviewed the use of perine architecture as a taxonomic tool in the species delineation of *Azolla*.

ENDOSYMBIOTIC CYANOBACTERIA

The cyanobacterial endophytes of all *Azolla* species are members of the Nostocaceae family and are collectively referred to as *Anabaena azollae* Strasburger. In the past 5 years, many studies on the *Azolla-Anabaena* symbiosis have been concerned with determining whether the endosymbiont is the same in all *Azolla* species. Immunofluorescent labeling and lectin hemagglutination techniques have been extensively used in such studies. Molecular studies have also contributed to knowledge in this area, and are described in greater detail later.

Unfortunately, the taxonomic relationship between *Azolla* cyanobionts remains unclear because studies using the aforementioned techniques have yielded apparently conflicting results. While the results of immunofluorescence studies[80,81] suggest that the endosymbionts of all *Azolla* species are similar (if not identical), recent results from lectin hemagglutination studies indicate that the cyanobacteria are different in at least three *Azolla* species.[82,83] Using either technique, it could be shown that free-living isolates of *Anabaena azollae* express different surface proteins than do symbiotic *Anabaena azollae*. The specific results of these studies are discussed separately in the following sections.

Immunofluorescence Studies

Immunofluorescence techniques have only recently been used in the taxonomic classification of organisms. Indirect fluorescent antibody labeling was first used to demonstrate the antigenic similarity between *Azolla* cyanobionts. In this study, antibodies raised against *Anabaena azollae* freshly isolated from *A. caroliniana* leaf cavities cross-reacted strongly with cyanobacteria freshly isolated from *A. pinnata*.[81] In a later study, fluorescent antibodies directed against *Anabaena azollae* freshly isolated from *A. microphylla, A. caroliniana, A. filiculoides,* and two strains of *A. pinnata* were used to show that the endosymbionts of those species shared an identical, highly specific surface antigen. Furthermore, the antibodies against the endophytes of all 4 species reacted strongly with freshly isolated endophytes of 6 *Azolla* species from 32 samples which had been collected throughout the world.[80]

Both of the studies described above reported that antibodies raised against freshly isolated phycobionts reacted weakly or not at all with free-living isolates of *Anabaena azollae*.[80,81] In addition, these antibodies reacted poorly with numerous strains of *Anabaena*,[80,81] two strains of *Nostoc*,[80] and one each of *Cylindrospermum*,[81] *Gleotrichia*,[80] and *Oscillatoria*.[80] Conversely, antibodies specific for the free-living *Anabaena azollae* isolate of Newton and Herman[47] failed to react with any of the freshly isolated symbiotic *Anabaena azollae*. These antibodies did, however, react with *Anabaena variabilis (Anabaena flos-aquae)*[80,81] and a free-living strain that was also isolated from *A. caroliniana*.[81] The results of these studies were substantiated by cross-absorption tests.[80,81] It has also been reported that a free-living culture of *Anabaena azollae* that was originally isolated from *A. filiculoides* is immunologically distinct from its symbiotic counterpart.[84]

More recent studies[85] have used indirect fluorescent antibody labeling to show antigenic cross-reactivity between freshly isolated *Anabaena azollae* and extracts of endophyte-free plants. This cross-reaction could not be demonstrated in Newton's free-living isolate.

Immunoassays have also shown that antibodies raised against free-living *Anabaena azollae* that was originally isolated from *A. caroliniana* cross-reacted with free-living isolates from two New World species of *Azolla*.[81,84] Curiously, these antibodies did not react with two free-living species originally isolated from *A. pinnata*, an Old World species.[80,84]

In summary, the results of immunofluorescence studies indicate that there are no detectable antigenic differences between phycobionts freshly isolated from leaf cavities of *A. caroliniana, A. mexicana, A. nilotica, A. pinnata, A. microphylla,* or *A. filiculoides*. However, these freshly isolated cyanobacteria are immunologically distinct from the free-living *Anabaena azollae* strains that have been tested.

To account for these results, it has been suggested that isolation and prolonged subculturing of *Anabaena azollae* results in either alteration of the surface antigens and morphology of the free-living isolates[80,81,84] or selection for a mutant.[81] It has also been suggested that free-living strains of *Anabaena azollae* may not be true isolates from the *Azolla* leaf cavity.[80,85] More recently, the results from restriction mapping studies[86] have indicated that Newton's isolate has different DNA hybridization banding patterns than those of *Anabaena azollae* freshly isolated from leaf cavities (see the section titled "Molecular Biology," subsection "Transcript Levels of Key Enzymes in *Anabaena azollae*", for a discussion of these results).

As noted by Ladha and Watanabe,[80] the probability is extremely low that spontaneous pleiotropic mutation in Newton's isolate could result in the expression of highly specific surface antigens identical to those of *Anabaena flos-aquae*. Likewise, it is improbable that mutation could account for the antigenic similarities between free-living isolates derived from different cultures of *Azolla*.

If the free-living isolates were not epiphytic contaminants, then the possibility that there is more than one population of cyanobacteria within an *Azolla* cavity should be seriously considered. Reestablishment of the symbiosis from the two free-living partners has not been achieved to date. In the absence of these data, the possibility remains that the major constituent of *Azolla* cavities is an obligate endosymbiont and that free-living isolates may be minor constituents of the cavity that are selected for during isolation. Investigations exploring this possibility are currently underway in our laboratory.

Lectin Studies

For a number of years, it has been postulated that lectins (phytohemagglutinins) play an important role in the recognition process between some symbiotic partners.[87] In the *Azolla-Anabaena* association, the necessity for recognition between the two partners has been questioned because the symbiotic state is maintained throughout the life cycles of both organisms.[80,85] Interactions between *Anabaena* filaments and cavity hairs lead to the enclosure of cyanobacterial cells within the developing cavity during the process of cavity infection. Lectins have been suggested to play a role in a recognition process between *Azolla* and *Anabaena*. Alternatively, it has been suggested that this partnership may simply result from the entrapment of cyanobacterial cells within mucilage, rather than from specific interactions.[17]

Nevertheless, conventional hemagglutination techniques have been used to demonstrate the presence of lectins in cyanobacteria freshly isolated from three *Azolla* species. Although it has been suggested that lectins are associated with the cyanobacterial partner,[88,89] the majority of reports have indicated that hemagglutinating activity is specifically associated with the fern.[82,83,85]

In contrast to results of immunofluorescence studies, results of lectin studies indicate that each *Azolla* species may be inhabited by different cyanobacterial populations. Most recently, McCowen et al.[82] demonstrated hemagglutinating activity of lectins isolated from both *A. pinnata* and *A. filiculoides*. Hemagglutinating activity could be inhibited by preadsorption of the *A. pinnata* lectin to cyanobacteria freshly isolated from *A. pinnata*, but not to cyanobacteria isolated from *A. filiculoides*. Likewise, the hemagglutinating activity of the *A. filiculoides* lectin could only be inhibited by preadsorption to cyanobacteria freshly isolated from *A. filiculoides*. Hemagglutination by either lectin could not be inhibited by preadsorption to free-living cyanobacterial isolates from the two fern species.

The aforementioned lectins[82] are apparently different from a lectin which was isolated from *A. caroliniana*.[83] The results of molecular weight determinations using gel filtration indicated that both the *A. pinnata* and *A. filiculoides* lectins had a subunit molecular weight of 5,000 Da.[82] In contast, the *A. caroliniana* lectin was reported to have a subunit molecular weight of 21,000 Da.[83] Furthermore, the inhibition of hemagglutinating activity by carbohydrates was species-specific. Whereas D-galactose specifically inhibited hemagglutinating activity of the *A. caroliniana* lectin, it was not inhibitory to the *A. filiculoides* lectin and was only slightly inhibitory to the *A. pinnata* lectin. Hemagglutinating activity of the *A. filiculoides* lectin was specifically inhibited by D-fructose. Other studies have shown that hemagglutinating activity of Newton's isolate[47] is specifically inhibited by D-fucose and L-rhamnose.[88,89]

Notably, the amount of lectin produced appears to be dependent upon growth conditions. In *A. caroliniana*, the lectin titer decreased when plants were grown on media containing nitrate.[83] Therefore, if lectins do play a role in a recognition process between *Azolla* and *Anabaena*, further attempts to reinfect endophyte-free *Azolla* with cyanobacteria might be facilitated by using plants subjected to nitrogen limitation either before or during the experiment.

ENVIRONMENTAL FACTORS AFFECTING THE GROWTH OF *AZOLLA*

The major growth requirements of the *Azolla-Anabaena* symbiosis are water, inorganic nutrients, air, and light. The interrelationships between a number of environmental parameters that affect the growth rate of *Azolla* are illustrated in Figure 13. Factors having significant effects on growth rates include pH, light quality and quantity, temperature, plant density, sporulation, water turbulence, and humidity.

The following sections describe the effects of a few of these environmental parameters on the physiological processes of *Azolla*. The complex interactions between such parameters are reflected in the apparent discrepancies between the results of different studies. In addition, while only one variable is usually changed at one time in controlled laboratory experiments, a number of variables change concomitantly during environmental studies. It should also be realized that extreme environmental conditions are frequently transient. For instance, plants which are incapable of withstanding prolonged periods of high temperature or light intensity under laboratory conditions may be capable of withstanding them for short periods of time during the diurnal cycle. Thus, it is not surprising that diverse results have been reported.

WATER

As discussed in the following chapter, the unavailability of water is one of the major constraints to more widespread use of *Azolla* as a biofertilizer. *Azolla* consists of 90 to 95% water,[90,91] which is required for maintenance of structural integrity and physiological processes. Although plants may become rooted either in shallow water or on the surface of moist mud,[54,58,60,92] they normally grow in the free-floating state. Therefore, they are quite susceptible to fragmentation caused by wind and wave action. Fragmentation has been shown to result in decreased rates of growth and nitrogen fixation.[58,93] The adverse effect of water turbulence was demonstrated by the observation that *Azolla* plants that were transferred prior to acetylene reduction measurements exhibited lower rates than undisturbed plants.[58]

Water depth can also influence the growth rate of *Azolla*. A water depth of 3 to 5 cm is recommended for optimal growth of *Azolla* in both nature[54] and the laboratory.[5]

Lastly, *Azolla* is very sensitive to desiccation and requires a minimum relative humidity of 60% for growth.[54,58] The optimum relative humidity is 85 to 90%.[54] If desiccation does occur, growth and nitrogen-fixing activity are irreversibly inhibited within 24 h.[94]

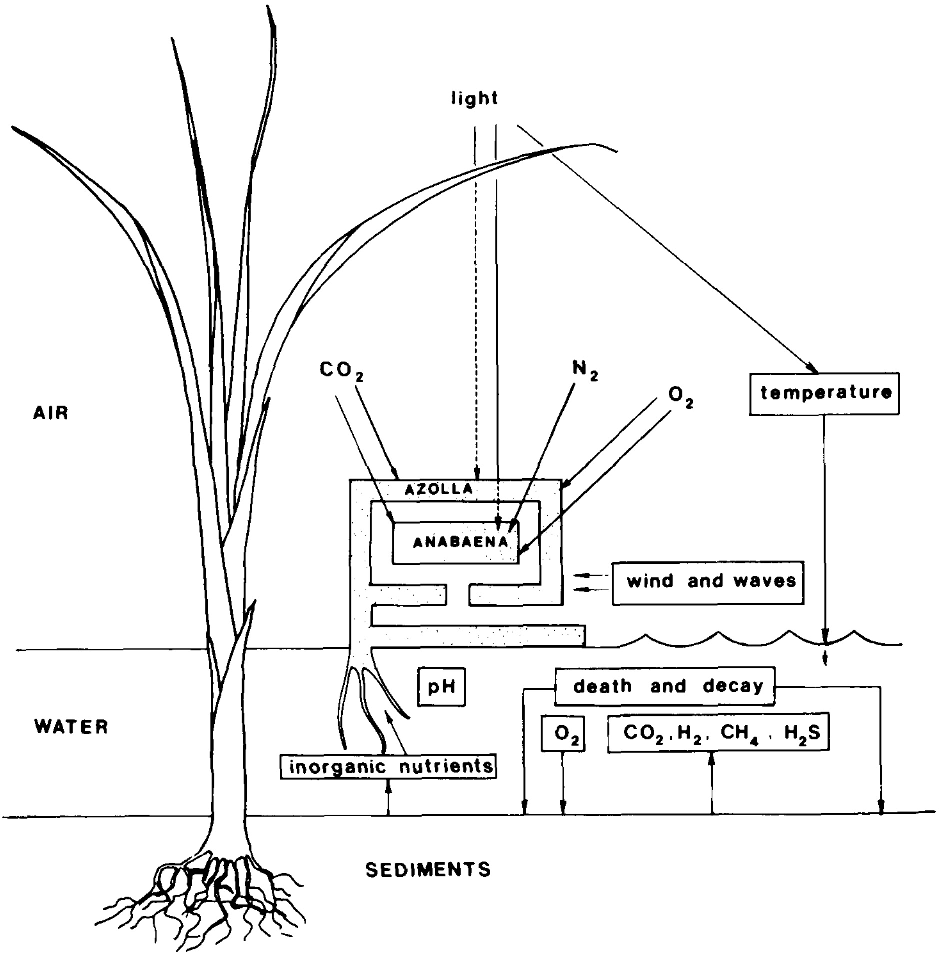


FIGURE 13. Schematic drawing depicting the environmental parameters having an effect on the growth rate of *Azolla*. (From Becking, J. H., in *Nitrogen and Rice*, International Rice Research Institute, Los Banõs, Philippines, 1979, 345. With permission.)

DISTRIBUTION

Because turbulence has a detrimental effect on the growth of *Azolla* sporophytes, they are not indigenous to turbulent waters. Instead, they are most commonly found on the surface of lakes, slow-moving rivers, canals, ponds, ditches, and rice paddies in regions of temperate and tropical to subtropical climate.

The natural global distribution of *Azolla* spp. has been described.[1,70] In general, *A. caroliniana*, *A. filiculoides*, *A. mexicana*, and *A. microphylla* were native to the Americas, while the distribution of *A. pinnata* was confined to the Western Hemisphere. *A. rubra* was found only in certain areas of Japan, Korea, Australia, and New Zealand, and *A. microphylla* was found only in the Galapagos Islands. *A. nilotica* was found within a defined area of Africa. However, as discussed by Lumpkin and Plucknett,[1,70] dispersal of *Azolla* (primarily by man) has resulted in considerable changes in the geographic distribution of *Azolla* species.

MEDIA

Growth of *Azolla* can be attained on a variety of mineral media,[5,58,60] some of which have been summarized and characterized with respect to anion and cation content by Becking.[58] The effects of different mineral media on the growth rate of *Azolla* were examined in a

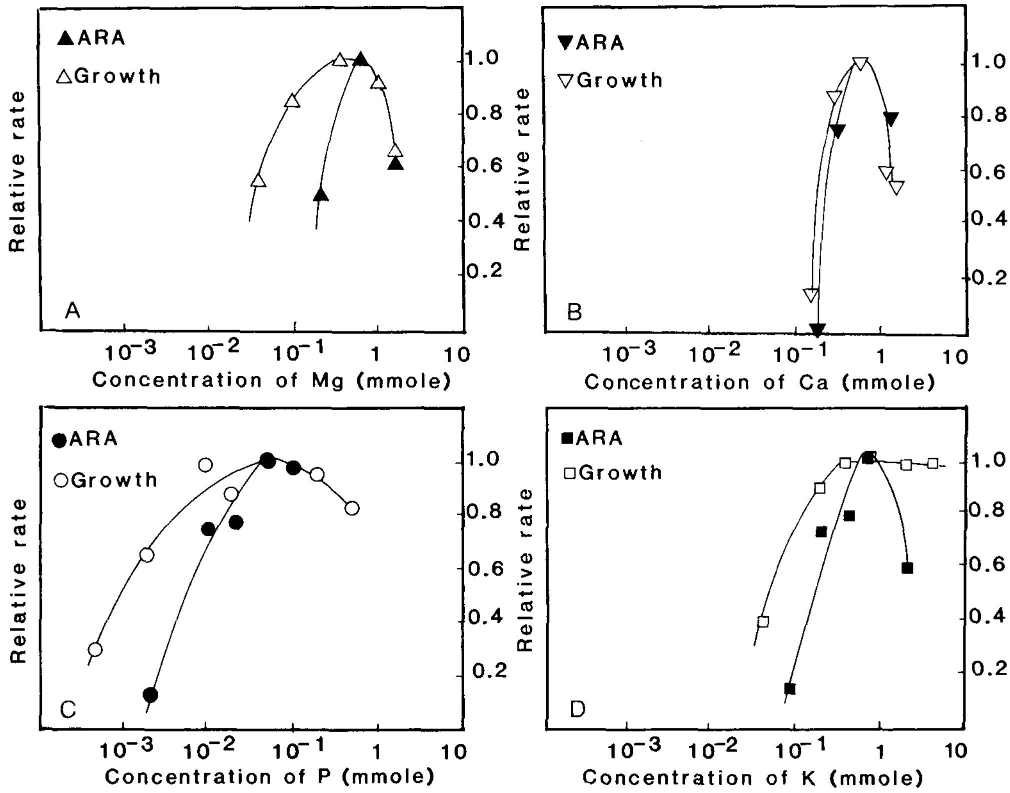


FIGURE 14. Effect of macronutrient concentration on rates of growth (open symbols) and acetylene reduction activity (closed symbols) in *A. pinnata*. Concentrations of (A) Mg, (B) Ca, (C) P, and (D) K, are shown in millimoles per liter nutrient solution. (Data adapted from Yatazawa, et al.[96])

recent study.[5] No pronounced effect on the growth rate of four *Azolla* species was observed on 20%, 40%, or 80% Hoaglands medium, IRRI medium, or a composite medium. When grown on IRRI medium, the growth rate, nitrogen content, and acetylene reduction rates were either the same as or slightly higher than when grown on the other media. The addition of a buffer had no effect on the doubling time of *Azolla*, which was approximately 2 d for all species. In the absence of a buffer, however, the media pH decreased with time, resulting in decreased growth rates.[5] Therefore, either daily pH adjustment or frequent transfer of cultures in the absence of buffer has been recommended.[5,95]

MINERAL REQUIREMENTS

Growth of *Azolla* is dependent upon the presence of minimum concentrations of key nutrients. The most important macronutrients are K, Ca, Mg, and P, which reportedly comprise approximately 1.0 to 2.0, 0.5, 0.5, and 0.5% (dry weight) of an *Azolla* plant, respectively.[54] The effect of these elements on rates of growth and acetylene reduction of *Azolla* is presented in Figure 14. These data have been adapted from Yatazawa et al.,[96] who reported the effect of nutrient deficiencies on the growth and nitrogen-fixation rates of *Azolla pinnata* var. *imbricata*. In this study, the threshold concentrations of P, K, Mg, and Ca were approximately 0.03, 0.4, 0.4, and 0.5 mmol/l for growth and 0.03, 0.6, 0.5, and 0.5 mmol/l for nitrogen fixation, respectively. As reported in other studies, Ca deficiency produced the most severe symptoms,[50,96,97] followed by deficiencies in either P[50,97] or Mg.[96] The appearance of *Azolla* under conditions of nutrient deficiencies and the minimum concentrations of these nutrients required for growth of *Azolla* in the field are addressed in the following chapter.

The effects of micronutrient deficiencies on the growth and nitrogen-fixation rates of *Azolla* have also been examined.[96] As might be expected, a deficiency in the micronutrients Fe, Mn, Mb, Co, B, Zn, or Cu had unfavorable effects on the growth of *Azolla*.[96] It was shown that micronutrient deficiency was most likely to occur for Fe, Mn, Mo, or B. However, absolute deficiencies in many instances may be impossible to achieve because micronutrients frequently occur as contaminants of water and other minerals. In this study,[96] the threshold concentrations of Fe, Mn, Mo, and B were approximately 50, 20, 0.3, and 30 μg/l for growth and 20, 10, 1, and 20 μg/l for nitrogenase activity, respectively. Other reports have indicated that Na,[98] Co,[98,99] and Mo[16,98] are specifically required by *Anabaena*.

Inadequate concentrations of P or Fe most frequently limit the growth of *Azolla* in nature.[95,100,101] Phosphorous is usually the limiting nutrient in rice paddies.[1,100] Methods used in the application of P fertilizers to rice paddies are discussed in detail in the following chapter.

EFFECT OF pH

As is true for most biological systems, growth of the *Azolla-Anabaena* symbiosis is limited at extreme pH values; optimal growth of most species occurs between pH 5 and pH 8.[5,16,50,60,92,109] Under laboratory conditions, comparable rates of growth and nitrogen fixation were reported for *A. caroliniana, A. mexicana, A. filiculoides,* and *A. pinnata* on mineral media with and without a buffer over a pH range of 5 to 8.[5] Decreased growth rates occurred above pH 9 or with increased culture age due to a drop in the pH of the medium.[5,60] In another laboratory study, *A. mexicana* grew at comparable rates over a pH range of 4.2 to 8.0.[60] Watanabe et al.[97] and Singh[92] have reported the pH optima for growth of *A. pinnata* to be 5.5 and 6, respectively.

The fact that pH has a considerable effect on the solubility of micronutrients and phosphorous is well known. Therefore, decreased rates of growth at high pH values may be a function of nutrient inavailability. In particular, the inavailability of iron due to its precipitation in neutral or alkaline solutions has resulted in decreased growth rates of *Azolla*.[5,96,97,109] This was demonstrated in an experiment in which iron-deficient *Azolla* was transferred to Fe^{+3}-amended media with a pH of either 4.0 or 7.0. At pH 4.0, the plants exhibited high rates of growth and nitrogenase activity. Plants transferred to pH 7.0 medium, however, showed a further decrease in growth.[96] The growth and distribution of *Azolla* in natural environments is also affected by pH and available iron concentration.[98,103]

Complex interactions between pH, light intensity, and/or temperature and the effect these have on ubiquitous organisms have also been reported to affect the growth rate of *Azolla*.[93,109] In a study conducted by Tuan and Thuyet,[109] it was reported that optimal growth of *Azolla* occurred at pH 4.5 to 5.5 However, the pH of paddy water increased between morning and noon due to photosynthesis by algae. At the higher pH, CO_2 disappeared, concentrations of bicarbonate decreased, and the carbonate ion concentration increased. If algal photosynthesis was inhibited either by shading or through the addition of copper sulfate, then the pH of the paddy water remained relatively constant and was more favorable for growth of *Azolla*.

Therefore, an additional consideration is that organisms which are ubiquitous to a given environment may influence the growth rate of *Azolla*. For instance, competition from non-nitrogen-fixing organisms has been shown to have a detrimental effect on the growth rate of *Azolla* in the presence of high concentrations of combined nitrogen.[1,97]

EFFECT OF TEMPERATURE

Temperature is clearly one of the most important environmental variables governing the growth and distribution of *Azolla* in aquatic environments. Numerous studies have examined the effect of temperature on the growth and/or nitrogen-fixation rates of *Azolla*. Results from several of these studies are presented in Table 2. It is difficult to make direct comparisons between individual studies because these studies vary considerably with respect to the strains used, light intensity, photoperiod, nutrient conditions, length of experiment, and time allowed for the acclimatization of plants prior to testing. In addition, it is difficult to determine the effect that any one factor has on growth because light intensity,[104,105,109] pH,[109] and nitrogen source[93] appear to interact with temperature to influence the growth rate of *Azolla*.

The studies discussed below have tested the effect of temperature on at least four species of *Azolla* concomitantly.[5,100,106] Two of these studies were performed in the laboratory, while the third was performed under environmental conditions. The results from laboratory studies necessarily differ from those performed under environmental conditions, but they do give a general indication of the relative temperature tolerances of different *Azolla* species.

In a comprehensive laboratory study, Peters et al.[5] examined the effect of several environmental parameters, including temperature, on the growth rate, acetylene reduction rate, C to N ratio, percentage dry weight, and chlorophyll (Chl) *a* to *b* ratios of four *Azolla* species. The effect of constant temperature on growth of *A. pinnata, A. caroliniana, A. filiculoides,* and *A. mexicana* was examined between 15 to 40°C under a light intensity of 16 klx (obtained through combined fluorescent and incandescent lighting) with a 16/8-h day/night photoperiod. Maximum growth rates, nitrogenase activity, and nitrogen content occurred at 30°C for *A. mexicana* and *A. pinnata*, at 25 to 30°C for *A. caroliniana*, and at 25°C for *A. filiculoides*. Temperature optima were the same at pH 6 and pH 8, and temperature had little effect on the Chl *a* to *b* ratios below 35°C. The percentage dry weight and the C to N ratios changed dramatically with temperature and were higher at the temperature extremes (i.e., at 15 or 40°C) than at the optimum growth temperatures. Whereas none of the strains grew for prolonged periods of time at 40°C, the relative tolerance to this temperature was *A. mexicana* > *A. pinnata* > *A. caroliniana* > *A. filiculoides*.

In a second laboratory study,[100] the effect of temperature on the growth rates of two strains of *A. pinnata*, and one each of *A. mexicana, A. caroliniana, A. microphylla,* and *A. filiculoides,* was measured for 6 weeks at a combined fluorescent and incandescent light intensity of 20 klx with a 12-h photoperiod. Plants grown under a 28/20°C (day/night) regime were subjected to a thermoperiod of 37/29°C, and their response with respect to growth rate, rate of acetylene reduction, total protein, and chlorophyll content was recorded. Under these conditions, *A. filiculoides* stopped growing within 4 weeks, and the endophyte was absent after 2 weeks. The doubling time of the *A. pinnata* strains increased from approximately 2.3 to 3.5 d, whereas cultures of *A. microphylla* required almost twice as much time to double in biomass. Because the doubling times of *A. caroliniana* and *A. filiculoides* were greatly increased, these species were considered to be heat sensitive. Decreased amounts of protein and chlorophyll were observed in these heat-sensitive species. In general, heterocyst frequencies and rates of acetylene reduction paralleled growth rates.

In summary, the results of these studies indicated that the relative heat tolerance of these strains to a 37/29°C thermoperiod under the conditions employed was *A. pinnata* > *A. microphylla* > *A. mexicana* > *A. caroliniana* > *A. filiculoides*.[100]

A predictive model for the environmental growth response of *Azolla* has also been reported.[106] The relative growth rates of the seven extant species of *Azolla*[1,61,66] were recorded over a 1-year period in China. Temperatures ranged from −7°C in January to 38°C in July; the mean temperatures for these months were 4.4 and 27.9°C, respectively. Cultures were grown on nitrogen-free defined medium under glass, but were otherwise subject to ambient environmental conditions. *A. caroliniana, A. filiculoides,* and *A. pinnata* var. *imbricata* were the only species that survived most of the year. Accessions of *A. rubra* and *A. pinnata* var. *pinnata* died during the warmer months, whereas those of *A. mexicana, A. microphylla, A. pinnata* var. *pinnata*, and *A. nilotica* died during colder months.

Two distinct peaks in the maximum relative growth rate, corresponding to the spring

TABLE 2
Effects of Temperature and Light Intensity on *Azolla*

Species	Location	Growth conditions	Temperature	Light intensity[a,b]	Notes	Ref.
A. pinnata	Ranchi, India	Tap water and garden soil in pots	Ambient; February 1 to March 2, 1981	80 klx — highest moisture content: fresh and dry weights all decrease with decrease in LI 40 klx — maximum area covered by fronds	30-Day test period	57
A. pinnata	Cuttack, India	Concrete tanks containing soil and water, or in field with 5—10 cm standing water	14—40°C growth observed, but better growth at 20—30°C		Phosphorous fertilizer added	50, 92
A. pinnata	Java, Indonesia	Mineral medium in pots	C_2H_2 reduction rate increased with temp up to 40°C, decrease noted at 43°C	16% sunlight for 5 h — get 15% decrease in C_2H_2 reduction rate; 16% sunlight for 10 hours — get 70% decrease in C_2H_2 reduction rate	Temperature is more important than light intensity in determining C_2H_2 reduction rates	91
A. pinnata	Ranchi, India	Tap water and garden soil in pots	Ambient; January 1 to 31st, 1982	Natural sunlight during day with incandescent light supplement where needed (150 W)	Highest fresh and dry mass, moisture content, thicker roots, using 12-h photoperiod; measured 24/0, 16/8, 12/12, 8/16, and 6/24 day/night	59
A. pinnata	Java, Indonesia			80—90 klx — C_2H_2 reduction rate is considerably reduced; shading 50—75% sun does not interfere with C_2H_2 reduction rates or photosynthesis	Lower LI experienced under rice cover favors N_2ase activity; depends on rice plant density and development	58

A. pinnata	IRRI, Los Baños, Philippines	Greenhouse, mineral medium	26/18°, 29/31°, 32/24°C — fresh weight and nitrogen accumulation about the same; 35/27°C — growth and nitrogen accumulation decreased to about 50% rate at other temperatures	Ambient	2-week study, 12/12-h photoperiod; at temp >26/18°C, smaller fronds with yellowish-brown color	97
A. pinnata	W. Java, Indonesia	Open field		100% sunlight optimal for biomass production		108
A. pinnata			5—40°C growth range			54
A. mexicana				50% sunlight or greater required for maximum biomass and nitrogen yield		93
A. mexicana	Illinois	Growth chambers, polyethylene containers partially covered with plate glass in growth chambers	23/18°C, optimum at 12 klx; grows well up to 30°C at LI <13 klx, but at rate $^1/_2$ that at 21°C; dies at 40°C; viable but no growth at 5°C	12 klx optimum at 23/18°C thermoperiod; 20 klx maximum for growth; 0.75 klx minimum for growth	Photoperiod 14/10, fluorescent lights	60
		Ambient (pond)	15°C — critical temp for germination and growth; 18—25°C — growth observed; >25°C — plants observed only in shade	95—120 klx — senescence when water temp >25°C	High light intensity most responsible for senescence of fern; no interaction seen between pH and light intensity	
A. filiculoides				C_2H_2 reduction and growth rates increase with light intensity up to 49 klx		93
A. filiculoides	S. Africa		27.5°C optimum; 5—45°C growth range			12

TABLE 2 (continued)
Effects of Temperature and Light Intensity on *Azolla*

Species	Location	Growth conditions	Temperature	Light intensity[a,b]	Notes	Ref.
A. filiculoides	California	Ambient	Frost tolerant; 10°C — nearly no growth or C_2H_2 reduction; 10—25°C — growth and C_2H_2 reduction increase exponentially; >30°C — growth and C_2H_2 reduction decline; prolonged exposure to 40°C results in death	64 klx optimum growth and C_2H_2 reduction rates; no decrease in either rate at 160 klx		101
A. filiculoides		Laboratory		40—80 klx growth rate same when temperature >25°C	Artificial light	105
A. filiculoides		Laboratory	Grown at 15, 20, 30°C; maximum C_2H_2 reduction rate at 30, 35, 40°C, respectively		Only for short periods of time	101
A. pinnata		Laboratory		At 14 klx and 27 klx C_2H_2 reduction rates are the same	Artificial light	58
A. caroliniana		Laboratory		4.8 klx — C_2H_4 reduction rates saturated; 2.15 klx — C_2H_2 reduction rate saturated in isolated *Anabaena*	Artificial light	35

[a] Light intensities were calculated based on the following conversions: 1 klx = 92.6 footcandles = 12.5 μmol $m^{-2}s^{-1}$ = 12.5 $\mu Em^{-2}s^{-1}$.

[b] LI = Light intensity.

and fall months, were observed during the aforementioned study. The results during these months were different enough from those obtained during the summer to warrant separation of the data prior to analysis. The relative growth rates during the spring and fall were *A. filiculoides* > *A. caroliniana* > *A. pinnata* var. *imbricata* > *A. rubra*.[106] During the summer months, the relative growth rates of all seven species were adversely affected by fungal growth. Therefore, the potential productivity was determined by relative growth rate, susceptibility to disease, and heat stress. It was found that *A. microphylla* and *A. pinnata* var. *imbricata* were relatively disease-free, had the highest relative growth rates, and were the most capable of tolerating high summer water temperatures. The relative growth rate of *A. pinnata* var. *imbricata* was also highly correlated with irradiance. During the summer months, *A. caroliniana, A. filiculoides,* and *A. nilotica* exhibited lower productivity which was presumably due to inhibition of growth by high water temperatures. *A. mexicana* exhibited a low relative growth rate because it frequently sporulated under these environmental conditions.

The authors concluded that, in the spring and fall, the relative growth rates of *Azolla* species were most closely related to minimum water temperature. During the summer months, however, growth rates correlated best with minimum water temperature, solar radiation, and relative humidity.[106]

The relative tolerances of *Azolla* species to temperature have been summarized in one report as follows: *A. filiculoides* and *A. rubra* are cold tolerant and heat sensitive, *A. microphylla* and *A. mexicana* are heat tolerant and cold sensitive, *A. caroliniana* and *A. pinnata* var. *imbricata* are relatively tolerant to both cold and heat, and *A. pinnata* var. *pinnata* and *A. nilotica* are neither cold nor heat tolerant.[107]

It should also be noted that acclimatization of *Azolla* plants can occur. It has been shown that, when plants were grown at low temperature and then subjected to a stepwise increase in temperature, nitrogenase activity increased with temperature up to 40°C and remained high even at 45°C.[105]

In conclusion, although individual reports vary considerably, it is clear that *A. pinnata* var. *imbricata, A. mexicana,* and *A. microphylla* are more tolerant to higher temperatures than are either *A. caroliniana* or *A. filiculoides,* and that *A. filiculoides* is most sensitive to prolonged exposure to high temperatures.

EFFECT OF LIGHT INTENSITY

Light intensity and photoperiod greatly affect rates of growth and nitrogen fixation in *Azolla*. In one report, high light intensity was considered to be the factor that was most responsible for senescence of the fern.[60]

The results of several studies examining the effects of light intensity or photoperiod on rates of growth and nitrogen fixation in *Azolla* are summarized in Table 2.

Several studies have reported that *Azolla* attains maximum biomass at light intensities greater than 50% sunlight.[57,93,108] In contrast, exposure to high light intensities usually resulted in decreased rates of nitrogen fixation.[57,58,60,93,94] Therefore, maximum growth rates of *Azolla* are generally attained at light intensities that are inhibitory to rates of nitrogen fixation. This observation has been substantiated by laboratory experiments which showed that nitrogenase activity was saturated at a light intensity less than 50% of that required to saturate rates of growth.[5] It should also be noted that the quality and quantity of light used in laboratory experiments are considerably different from those experienced in nature. In lab studies, therefore, rates of growth and acetylene reduction were saturated at light intensities that were much lower than those required in the environment.

As can be seen in Table 2, most but not all[82,86] reports are consistent with these observations. As suggested by Watanabe,[97] discrepancies between results may arise from variations in environmental conditions (e.g., temperature or plant density).

Finally, the effect of light intensity on rates of nitrogen fixation in the *A. caroliniana* association and the isolated endophyte have been measured.[35] It was found that rates of acetylene reduction were saturated at approximately 4.8 klx in the association and at approximately 2.1 klx in the isolated endophyte. The lower saturation point of the isolated endophyte might be expected because light is filtered through fern leaves before reaching the cyanobacteria within the leaf cavities.

Shading

There is a dual effect of temperature and light intensity on the physiological processes of *Azolla* under conditions of shading. In Western Africa, *Azolla* inocula must be grown under cover to survive the high light intensity.[94] Other reports have indicated that shading does not adversely affect rates of growth and/or nitrogen fixation in *Azolla*.[58,94,108] In one study, it was reported that up to 75% shading of sunlight could occur without interfering with rates of photosynthesis and nitrogen fixation.[58] The results of a different study[91] indicated that shading of 16 to 57% full sunlight for 5 h had no detrimental effects on the nitrogenase activity of *A. pinnata*. It was only when shading was increased to 16% full sun for 10 h that the nitrogen-fixation rate of *A. pinnata* was decreased to 30% of the level in control plants. In this study,[91] it was concluded that temperature, rather than light intensity, affected the rate of nitrogen fixation. The effect of complete darkness on the rate of nitrogen fixation has been discussed further in subsection ''Dependence of Nitrogen Fixation on Photosynthesis,'' in Section ''Nitrogen.''

Photoperiod

Photoperiod also affects both rates of growth and of nitrogen fixation. Peters et al.[5] reported that the growth rate of four *Azolla* species increased with increasing day length up to 24 h, whereas acetylene reduction measurements were usually highest under a 16/8-h light/dark photoperiod. In contrast, the nitrogen-fixation rate of *A. filiculoides* was reported to be saturated at a day length of approximately 8 h.[93] Photoperiod also has a pronounced effect upon growth of *A. pinnata*[57] (Table 2).

ADDITIONAL FACTORS AFFECTING GROWTH OF *AZOLLA*

In addition to the factors described above, rates of *Azolla* growth and nitrogen fixation are adversely affected by high plant densities.[54,58,60] Watanabe[54] noted that higher light intensities were required to attain maximum rates of nitrogen fixation under crowded conditions due to the overlapping of *Azolla* fronds.

For optimum growth in the laboratory, gaseous exchange should not be hindered by tightly stoppering flasks.[12] Peters et al.[5] reported that the introduction of humidified air into cultures was necessary to maintain the pCO_2 required to achieve maximum growth rates.

Plants that are undergoing sporulation exhibit decreased rates of growth.[93,106] This decrease is most likely due to the consumption of photosynthate that is required for sporulation.[106] Salinity may also affect the growth rate of *Azolla*.[1,70] Finally, under environmental conditions, major damage to *Azolla* crops can be caused by a variety of insects, fish, and fungi. These topics are discussed further in the following chapter.

PHYSIOLOGICAL STUDIES

Most of the physiological studies reported to date have used the *A. caroliniana* association. In an attempt to elucidate the contributions made by either partner of the symbiosis, results from studies on the intact association have often been compared to those obtained using endophyte-free plants and freshly isolated cyanobacteria. However, significant environmental changes are imposed upon the cyanobacteria by removal from the specialized niche that they occupy within the *Azolla* leaf cavity. Therefore, it is unlikely that the physiological characteristics of isolated *Anabaena azollae* accurately reflect the *in situ* physiological characteristics of the organism. In addition, the applicability of studies which have used free-living isolates of *Anabaena azollae* should be questioned because free-living isolates have not yet been shown to be the major constituent of the *Azolla* leaf cavity. Lastly, the pH, concentration of O_2 and other gases, and the amount of light within the cavity have not yet been determined.

The evaluation of this system is complicated even further because the cavity milieu changes with leaf age. During the aging process, populations of cavity hairs, cyanobacteria, and bacteria increase with leaf age. The physiological capabilities of the cyanobacteria have also been shown to change with leaf age. Thus, it is apparent that many basic questions concerning the ontogenic physiological changes which are imposed on *Anabaena* by the symbiotic state remain unanswered.

The following sections are designed to provide general background information on photosynthesis and nitrogen fixation in the *Azolla-Anabaena* symbiosis. For a more extensive review of physiological studies, the reader is referred to earlier review articles.[20,22,45,95,109-112]

SEPARATION METHODS

An accurate assessment of the relative roles played by either partner in the *Azolla-Anabaena* symbiosis is dependent upon effective separation of the two organisms prior to the study. The plants must also be freed of epiphytic contaminants, usually by washing *Azolla* with dilute solutions of sodium hypochlorite, with or without detergent.[11,18,47,49,99]

Because *Anabaena*-free *Azolla* occurs infrequently in nature,[16,26] several methods have been employed to generate *Anabaena*-free cultures for use in laboratory studies. *Azolla* fronds have reportedly been freed of cyanobacteria using methods such as antibiotic treatment,[12,58,113] changes in light intensity,[26,29] high temperatures,[100] cold treatment,[16] dilution of culture medium,[16] nitrogen starvation, and treatment with hypochlorite.[16] Endophyte-free cultures of both *A. caroliniana* and *A. filiculoides* have been obtained by regenerating new plants from apical tips which had been excised from surface-sterilized plants.[11] Most recently, it was reported that endophyte-free cultures can be generated by germinating fertile megasporocarps from which the apical cap had been removed.[75]

Endophyte-free *Azolla* cultures are necessarily grown in media containing a source of combined nitrogen. These plants, which are smaller and less green, exhibit less branching, have more roots, and grow slower than do endophyte-containing plants.[15,26,29,113] Proof that *Azolla* cultures truly lack the endophyte can be established by the absence of growth on media lacking a combined nitrogen source and the inability of these cultures to reduce acetylene.

Several researchers have reported the isolation and growth of cyanobacteria from *Azolla* sp.[12,14,47,81,84,114,115] In general, this is achieved by homogenization or microdissection of either surface-sterilized *Azolla* fronds or isolated algal packets. The liberated cyanobacteria are subsequently grown on dilute culture medium. As discussed previously (subsection "Endosymbiotic Cyanobacteria" in Section "Taxonomy"), however, there is no proof that these free-living isolates are identical to the major cyanobacterial endosymbiont found within *Azolla* leaf cavities.

Numerous *Azolla* fronds are gently squashed with a rolling pin to isolate large quantities of *Anabaena azollae*. After the cyanobacteria are liberated, they are separated from plant material by filtration and centrifugation. With this "gentle roller" technique,[113] it is possible to obtain nearly pure preparations of cyanobacteria for use in physiological and molecular studies.

PHOTOSYNTHESIS

One of the relatively unique properties of the *Azolla-Anabaena* symbiosis is the ability of both partners to fix atmospheric CO_2.[116] Photosynthetic CO_2 fixation and evolution of O_2 have been demonstrated in cyanobacterial populations freshly isolated from *Azolla* leaf cavities.[35,117] Furthermore, autoradiographic studies have shown that CO_2 fixation occurs exclusively within the vegetative cells of the endophyte.[35]

Photosynthetic Metabolism

Pulse-chase studies have demonstrated that both partners in the symbiosis fix CO_2 via the Calvin cycle.[117] Sucrose has been identified as the major product of photosynthesis in both symbiotic and endophyte-free *Azolla*. However, when $^{14}CO_2$ was added to *Anabaena azollae* that had been isolated from leaf cavities, ^{14}C-sucrose was not detected as a major product of cyanobacterial photosynthesis.[117] A more recent study has indicated that, if the intact association was pulsed with $^{14}CO_2$ and the cyanobacteria subsequently isolated, then a time-dependent accumulation of ^{14}C-sucrose occurred in the endophyte.[45] These findings show that sucrose that is synthesized by *Azolla* is transferred to the endosymbiont.

Evidence for the cross-feeding of carbon compounds has also been obtained from other experiments.[35,46] The process of nitrogen fixation is ultimately dependent upon photosynthesis for a source of reductant and ATP (see next Section). The observation that a long period of time elapsed before rates of acetylene reduction were inhibited following the addition of DCMU implies that the cyanobacteria receive photosynthate from *Azolla* during this lag period.[46]

It is believed that cyanobacterial CO_2 fixation occurs primarily within vegetative cells located at the apical meristem of *Azolla*,[26] and that *Anabaena* changes to a mixotrophic[35] or photoheterotrophic[46] type of metabolism in older leaf cavities. The number of heterocysts in symbiotic *Anabaena azollae* has been shown to increase with leaf age.[26,29] Furthermore, heterocysts lack photosystem II (PS II) activity and are incapable of CO_2 fixation.[118] Therefore, this proposed shift in metabolism seems feasible because it would provide the larger amounts of reductant needed to support nitrogen fixation by the large heterocyst populations in the older leaf cavities.

Effects of Light Intensity, Photoperiod, and Temperature on Photosynthesis

The effects of light intensity, photoperiod, and temperature on the rate of CO_2 fixation have also been examined. It was shown that rates of CO_2 fixation were saturated in both *A. caroliniana* and the isolated endophyte at approximately 8 klx.[35] Studies examining the effect of temperature on $^{14}CO_2$ fixation rates have suggested that a temperature of 35°C is inhibitory to protein synthesis in *A. pinnata*.[119] On the other hand, maximum rates of CO_2 fixation were reported to occur at 36°C in the *A. caroliniana* endophyte.[110] In regard to the effect of photoperiod on various physiological processes, it was shown in *A. caroliniana* that rates of photosynthesis were approximately the same regardless of whether the day length was 12, 16, or 24 h.[20,112,120]

Photorespiration

The process of photorespiration has been studied in the *A. caroliniana* symbiosis.[117] When either the intact association or endophyte-free plants were assayed under atmospheric conditions, the CO_2 compensation point was approximately 40 μl/l. This high value is similar to those reported for other plants which utilize the Calvin cycle. The CO_2 compensation point was dependent upon the concentration of O_2 and was shown to decrease drastically when the O_2 concentration was decreased to 2%. Also, an increase in the concentration of O_2 from 2 to 20% resulted in a 40% inhibition of the rate of CO_2 fixation. These results indicated that *Azolla* carries out typical photorespiratory metabolism.

In contrast, the cyanobacterial CO_2 compensation point was less than 5 μl/l. This compensation point was insensitive to the concentration of O_2, and rates of CO_2 fixation and O_2 evolution were the same under aerobic or microaerobic conditions. This low compensation point is similar to those reported for plants lacking photorespiratory metabolism (e.g., corn). Therefore, as in other cyanobacteria,[121,122] photorespiratory metabolism is not detectable in *Anabaena azollae*.[117]

Photochemical Activity

Photochemical activity of both photosystems I and II has been demonstrated spectrophotometrically in chloroplasts from symbiotic and endophyte-free *A. caroliniana* and in cell-free extracts of the endosymbiont.[113] Estimates of PS I and PS II activities were obtained by estimating the chlorophyll to P700 ratio and delayed light emission, respectively. The results indicated that both PS I and PS II activities were lower in *Anabaena azollae* than in *Anabaena cylindrica*. When studied at the cellular level, it was determined that the chlorophyll to P700 ratio in *Anabaena azollae* was consistently higher in vegetative cells than in heterocysts. The results of delayed light emission studies indicated that PS II activity was approximately ten times greater in vegetative cells than in heterocysts. These results are in agreement with the observation that heterocysts lack PS II reaction centers.[118]

Pigments

The cyanobacterial pigments used for harvesting light energy for photosynthesis include chlorophyll *a*, phycobiliproteins, and carotenoids.[113,123,124] The complement of pigments produced by *Azolla* includes chlorophylls *a* and *b* and carotenoids.[123]

The cyanobacterial phycobiliproteins c-phycocyanin, allophycocyanin, and phycoerythrocyanin have been isolated and characterized from the *A. caroliniana* symbiosis.[125] The absorption and fluorescence emission spectra of the phycobiliproteins obtained from freshly isolated *Anabaena azollae* and free-living cyanobacteria have been compared. Results from these studies suggest that there may be some degree of chromatic adaptation in the pigments of the endophyte.[123,125-127] This might be expected because light reaching *Anabaena azollae* must first pass through the fern leaf.

The pigment content and composition of both *Azolla* and *Anabaena* is influenced by environmental conditions.[3,92,110,117] Anthocyanins, including luteolinidin-5-glucoside,[3,128] are produced by *Azolla* (except *A. nilotica*[4]) under conditions of high light intensity[16,54,60,68] or low temperature.[3,16] The cyanobacterial phycobiliprotein content is also reportedly variable with culture conditions.[110,117]

Absorption Spectra Measurements

Analysis of fluorescence and emission spectra indicates that the pigmentation of *Azolla* and *Anabaena* is complementary.[91,117,123,124] The cyanobacterial phycobiliproteins absorb light in an area of the spectrum where absorbance by the fern is minimal. The *in vivo* and *in vitro* absorption spectra for isolated *Anabaena azollae*, endophyte-free *Azolla*, and the association have been studied in the *A. caroliniana* symbiosis.[117,123,124] The results of these studies show that the *in vivo* spectra of the endophyte-free and symbiotic *Azolla* are virtually indistinguishable. In agreement with this, absorption by cyanobacterial phycobiliproteins was demonstrated in *Anabaena* only after it was isolated from the fern cavity.[117,123,124] When the endosymbiont was analyzed either *in vitro* or *in vivo*, this phycobiliprotein absorption peak occurred between 580 and 640 nm.[117]

Action Spectra Measurements

The results of photosynthetic action spectra measurements[117] in the *A. caroliniana* association were consistent with the results of absorption spectra measurements. The action spectra for photosynthetic CO_2 fixation and O_2 evolution were nearly identical in the association and endophyte-free plants. The maximum quantum yield occurred in the range of chlorophylls *a* and *b* absorbance (650 to 670 nm), and a typical "red drop" was observed to occur above 670 nm.[117] Cyanobacterial photosynthesis, which was necessarily measured in the isolated endophyte, was most efficient in an area of the spectrum where phycobiliproteins absorb maximally (580 to 640 nm) and was shown to decline above 640 nm.[117]

The extent to which *Anabaena* contributes to light harvesting and photosynthesis in the symbiosis has not yet been ascertained. However, it has been estimated that approximately 12 to 22% of the total protein and 11 to 21% of the total chlorophyll in the symbiosis is contributed by the phycobiont.[123] The actual contribution by the endophyte is expected to be less than this percentage, however, because (at least in the *A. caroliniana* symbiosis) up to 50% of the cyanobacterial cell population may be either heterocysts or akinetes,[35] which are incapable of photosynthesis.[117]

Occurrence of Phycobiliproteins in Heterocysts

In free-living, heterocystous cyanobacteria, phycobiliproteins are primarily found in vegetative cells, where they reportedly serve as the exclusive light-harvesting pigments for PS II.[118] In contrast, epifluorescence microscopy studies on the endophytes from *A. caroliniana*[124,129] and *A. filiculoides*[129] have suggested that phycocyanin contents are high within heterocysts of *Anabaena azollae.*

More recently, fluorescence emission microspectrofluorometry was used *in situ* to study the pigment composition of individual cyanobacterial cells that had been freshly isolated from sequential leaf cavities of *A. pinnata* and *A. caroliniana.*[32] These data confirmed the observation that heterocysts of symbiotic *Anabaena* have high phycocyanin contents. Additionally, they showed that the complement of phycobiliproteins in vegetative cells and heterocysts was similar, and did not change with leaf age. The phycobiliprotein content in heterocysts was also unaffected by leaf age.

It has been suggested that the occurrence of phycobiliproteins in heterocysts may result in a less favorable environment for nitrogenase activity.[29] In contrast, it has been argued that the retention of phycobiliproteins by heterocysts is a logical adaptation for *Anabaena azollae*, which is exposed only to light that has been filtered through a green leaf.[32] The function of these light-harvesting pigments in heterocysts of symbiotic *Anabaena azollae* is an area of current research interest.

NITROGEN

The ability of *Anabaena azollae* to fix atmospheric nitrogen allows *Azolla* to grow in areas that are naturally low in combined nitrogen. It is this attribute that makes this association an important biofertilizer in many areas of the world (see following chapter). It has been estimated that nitrogen comprises 3 to 6% of the dry weight of the association.[5,54,91,97]

Nitrogen fixation, which is carried out in heterocysts of the endophyte, supplies the total nitrogen requirement of both *Azolla* and *Anabaena.*[46] Because the number of heterocysts has been shown to increase with leaf age,[26,29] it is not surprising that nitrogenase activity shows a similar developmental profile. Some variability is encountered in the exact activity profile because of the different morphologies of *Azolla* species. Nevertheless, in *A. caroliniana,*[31,44,58,102] *A. filiculoides,*[26] and *A. pinnata,*[32,129] nitrogenase activity has been shown to increase from nearly zero at the shoot apex to a maximum level at approximately leaf number seven. Nitrogenase activity then levels off and declines in older leaves. In contrast to this profile, two peaks of nitrogenase activity were observed in one strain of *A. pinnata.*[31,129]

RATES OF NITROGEN FIXATION

Nitrogen fixation rates in *Azolla* have been estimated using assays for acetylene reduction, ^{15}N fixation, and ATP-dependent H_2 production. The results obtained from many of these reports have been summarized previously.[1,54]

It has been estimated that symbiotic *Anabaena azollae* reduces acetylene at rates that are 4 to 18 times greater than those observed in free-living *Anabaena cylindrica*.[127] The higher rates reported for symbiotic *Anabaena* are at least partially due to the higher heterocyst frequencies (up to 30%) that are formed by *Anabaena* when it is grown in the symbiotic state.[26,29,35]

As discussed previously, several environmental parameters (e.g., temperature, light quality and quantity, and water turbulence) have a considerable effect on the rate of nitrogen fixation in *Azolla*. Rates are also affected by the lighting conditions employed prior to assay and are species-specific.[46,102,129,130] In addition, the conversion ratio most often used to estimate rates of nitrogen fixation from acetylene reduction assays, three acetylene reduced to N_2 fixed, may not be accurate for *in vivo* assays of the *Azolla-Anabaena* symbiosis. It has been shown, for example, that this ratio changes with pN_2 in the *A. caroliniana* symbiosis.[131] The results of several studies have suggested that this conversion factor may be lower in at least three of the *Azolla* species.[130,131]

HYDROGENASE

Data obtained from both laboratory[131] and field[130] studies on H_2 production by *Azolla* have suggested the presence of an efficient, unidirectional ("uptake") hydrogenase in the endophyte. This hydrogenase presumably functions in the recycling of electrons and ATP by oxidizing the H_2 produced during nitrogen fixation. Hydrogen production has been shown to be mediate y nitrogenase and is ATP-dependent.[131,132] The effects of combined nitrogen and partial pressures of a number of gases on hydrogen production have been reported.[126,127,131,132]

DEPENDENCE OF NITROGEN FIXATION ON PHOTOSYNTHESIS

Under anaerobic conditions, acetylene reduction in *Azolla* is light-dependent and saturates at a combined fluorescent and incandescent light intensity of approximately 4.8 klx.[46] Reduction of acetylene in the dark is dependent upon oxygen and pools of photosynthate as a substrate for respiration. In the dark, acetylene reduction proceeds only until the supply of photosynthate previously generated through photosynthesis and cyclic photophosphorylation is depleted.[46,102]

The indirect dependence of nitrogen fixation upon photosystem II activity has been clearly demonstrated in studies using the inhibitor DCMU.[46,102,124] If photosynthetic reserves were maintained by incubation of *Azolla* cultures in the light, rates of acetylene reduction were only inhibited by approximately 30% after the addition of DCMU. However, if pools of photosynthate were depleted by incubating cultures in the dark for 16 h prior to the addition of DCMU, then the addition of DCMU caused an inhibition in the rates of acetylene reduction that was approximately parallel to the inhibition of CO_2 fixation.[46]

Under aerobic conditions, the rate of acetylene reduction in the dark was less than 40% of that observed in the light. Therefore, it was suggested that respiratory-driven nitrogenase activity may be ATP-limited.[102] Subsequent experiments showed that cyclic photophosphorylation associated with PS I supplies most of the ATP for nitrogenase activity.[102] Therefore, nitrogen fixation in *Azolla* is considered to be a PS I-linked process that is only indirectly dependent on PS II activity.[112]

ACTION SPECTRUM FOR NITROGENASE

In both symbiotic *Azolla* and the isolated endophyte, equivalent rates of acetylene reduction were observed in the regions of phycobiliprotein (580 to 640 nm) and chlorophyll a (650 to 670 nm) absorption.[124] These results indicated that light absorbed by phycobiliproteins was as effective as light absorbed by chlorophyll in the reduction of acetylene. In contrast, as mentioned earlier, the action spectrum for photosynthesis in the isolated endophyte exhibited a single peak of maximum quantum yield in the area of phycobiliprotein absorption.

METABOLISM OF NITROGEN

Nitrogenous Pools

It has been shown that intracellular ammonia pools of symbiotically associated *Azolla* are five to ten times greater than those of endophyte-free *Azolla*.[15] The concentrations of amino acids in the nitrogenous pools of symbiotic *A. caroliniana* and the isolated endophyte have also been determined. In the endophyte, the major amino acid constituents of the nitrogenous pools were glutamate, aspartate, and arginine.[45] Glutamine was only a minor constituent of the amino acid pool. In the intact association, the major constituents were ammonia, glutamine, glutamate, and an unknown amino acid[45] that was subsequently identified as *N*-γ-L-glutamyl-β-D-aminophenylpropanoic acid.[133] However, significant concentrations of this glutamate derivative were only detectable in the *A. caroliniana* association.

In addition, *Anabaena azollae* isolated from leaf cavities excreted approximately 50% of the nitrogen that was fixed.[102] The majority of nitrogen excreted was as ammonia, with only insignificant amounts of organic compounds being detected.[102,111,119,123] Pulse-chase studies on the intact association indicated that the free $^{15}NH_3$ pool was very small and that ^{15}N was rapidly used in the synthesis of amino acids and proteins.

In free-living *Anabaena* spp., there is a very uniform heterocyst spacing pattern in which only the smaller daughter cells from asymmetrical cell divisions become heterocysts.[34] Only 10% of the total cell population differentiates into heterocysts, with the spacing pattern such that every tenth cell is a heterocyst. It is thought that the heterocysts fix N_2, which is then converted by glutamine synthetase (GS) to glutamine. This glutamine is then transported from the heterocysts into neighboring vegetative cells, resulting in the formation of a glutamine concentration gradient along the filament. Once the glutamine concentration is sufficiently low, another vegetative cell can begin to differentiate into a heterocyst.

If the above hypothesis is true, concentrations of GS might be expected to be low in symbiotic *Anabaena azollae*, which differentiates high frequencies of heterocysts. Conversely, concentrations of GS might be expected to be higher in symbiotic *Anabaena azollae* because the heterocysts of free-living cyanobacteria have higher concentrations of GS than do vegetative cells.[32]

Therefore, it has been of considerable interest to study GS protein concentrations, activities, and transcript levels in *Anabaena azollae*. The results from such studies are described below.

Activities and Concentrations of Ammonia-Assimilating Enzymes

Glutamine Synthetase

It has been determined that only 10% of the GS activity in the *Azolla-Anabaena* association is attributable to the endosymbiont.[123] Radioimmunoassays have also indicated that the endophyte contains approximately 5 to 10% of the GS protein that is found in free-living *Anabaena*.[134] In apparent contrast to this, however, the specific activity of GS in crude lysates of the endosymbiont was reported to be approximately 50% of that observed in free-living *Anabaena* species.[123] It has been suggested that the discrepancy between these results might be attributable to the fact that different normalization procedures were used. In the first study,[123] results were normalized with respect to phycocyanin content in the endophyte, whereas results of the latter study[134] were normalized with respect to diaminopimelic acid content of the cyanobacteria. Regardless of absolute values, both of these studies[123,134] indicated that GS is present in lower concentration in symbiotic vs. free-living *Anabaena*. Results of the molecular studies that examined GS transcript levels in symbiotic and free-living *Anabaena* spp. are discussed in the Section on molecular biology.

Glutamate Dehydrogenase and Glutamate Synthase

The activities of the ammonia-assimilating enzymes glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) have also been examined.[45,123,135] These studies indicated that the ammonia-assimilating enzymes are primarily associated with *Azolla* rather than with *Anabaena*. More specifically, it was shown that activities of GS and both NADH- and NADPH-dependent GDH were higher in cavity hairs than in *Azolla* leaves.[135] Inhibition studies using L-methionine-DL-sulfoximine (MSX) and ^{13}N indicated that ammonia is assimilated via the GS-GOGAT pathway in the endophyte, with no significant contribution by GDH or alanine dehydrogenase.[45]

Therefore, in the *Azolla-Anabaena* association, nitrogen fixed by *Anabaena* is transferred to *Azolla* where it is subsequently metabolized via the GS-GOGAT pathway.

TRANSPORT OF FIXED NITROGEN

When main stem axes of *A. caroliniana* were subjected to a pulse of $^{15}N_2$ and then sequentially dissected after increasing periods of time, the pattern of labeling clearly showed a time-dependent transport of ^{15}N from mature cavities to the stem apex.[44] This was consistent with observations that the nitrogen content and percent dry matter decreased, while the C to N ratio increased, with leaf age.[44] More recently, results of [$^{13}N/N_2$] pulse-chase studies suggested that the major compounds that are transported from the nitrogen-fixing cavities to the stem apex in *A. caroliniana* are glutamate, glutamine, ammonia, and possibly a glutamate derivative.[45]

EFFECTS OF COMBINED NITROGEN

Growth

Most laboratory studies have indicated that the growth rate of *Azolla* is not greatly affected by the presence of combined nitrogen.[15,20,29,54] The growth rates of *A. caroliniana, A. mexicana, A. filiculoides,* and *A. pinnata* were shown to be relatively unaffected by exposure to 5 m*M* ammonium, 25 m*M* nitrate, or 12.5 m*M* urea over a 5-week period.[20] A concentration-dependent increase in the percentage of daily nitrogen input from ammonium, nitrate, or urea was noted in all four species. The effects of combined nitrogen on the percentages of carbon, nitrogen, and dry matter were also determined in this study.[20]

The effect of combined nitrogen on growth of *Azolla* in the environment has also been studied. One report indicated that the effect of ammonium on rates of *Azolla* growth in rice paddies was dependent upon the ammonium salt used[137] (see Chapter 5).

As previously noted, combined nitrogen may have an indirect deleterious effect on the growth rate of *Azolla* by favoring growth of green algae that compete with *Azolla* for nutrients. Furthermore, growth of ubiquitous organisms may change the environment such that it is no longer favorable for growth of *Azolla*.[70,96,97]

Nitrogen Fixation

Both symbiotic *Azolla* and the isolated endophyte carry out nitrogen fixation at significant rates in the presence of combined nitrogen. Peters[35] reported that in the isolated endophyte, the addition of 10 m*M* ammonium chloride was required before inhibition of acetylene reduction rates was detectable. In contrast, acetylene reduction rates in *A. cylindrica* are reportedly 90% inhibited within 12 h by 1 m*M* ammonium.[138] These results suggest that ammonia concentrations within leaf cavities could be as high as 5m*M* without inhibiting nitrogen fixation rates. This is consistent with the observation that symbiotic *Azolla* contains high concentrations of ammonia.[15]

In regard to symbiotic *Azolla*, it was shown that rates of acetylene reduction in *A. caroliniana, A. filiculoides, A. mexicana,* and *A. pinnata* decreased with increasing concentrations of combined nitrogen.[20] However, these *Azolla* species retained 50 to 60% of the nitrogenase activity observed in control cultures after 5 weeks of growth in 5 m*M* ammonium. Growth in 12.5 m*M* urea resulted in a 50 to 60% decrease in rates of acetylene reduction in all four strains. The rates of acetylene reduction in *A. mexicana* were relatively unaffected by growth in 25 m*M* nitrate for 5 weeks, whereas *A. caroliniana, A. filiculoides,* and *A. pinnata* retained 40 to 60% of their nitrogenase activity.[20]

These results were in agreement with field studies that indicated that the addition of 25 kg N per hectare to rice paddies did not inhibit the process of heterocyst differentiation in *Anabaena azollae*.[92]

Inhibition of growth and/or nitrogen fixation of *Azolla* by the presence of combined nitrogen has also been reported.[70,91,96,97,136] The discrepancy between reports may be the result of strain specificity, variations in environmental conditions, and differences in concentrations of combined nitrogen.

The distribution of nitrate, ammonium, and dinitrogen in *A. pinnata* has been studied with radiolabeled nitrogen.[136] It was shown that nitrate-derived nitrogen was distributed primarily in older branches, whereas ammonium or dinitrogen-derived nitrogen was located primarily in younger branches. Therefore, it was proposed that the lesser effect of nitrate on rates of nitrogen fixation was due to its distribution in portions of *Azolla* that are not primarily concerned with nitrogen fixation.[136]

Finally, combined nitrogen has been reported to affect concentrations of chlorophyll[99] and rates of hydrogen production[91] in symbiotic *Azolla.*

MOLECULAR BIOLOGY

To date, only two molecular studies on the *Azolla-Anabaena* symbiosis have been described.[86,139] Both studies examined the cyanobacterial partner of this association at the molecular level.

ARRANGEMENT OF STRUCTURAL NITROGENASE (*nif*) GENES IN *ANABAENA AZOLLAE*

In a study of Franche and Cohen-Bazire,[139] the distribution of restriction sites around the *nif*HDK genes of endosymbionts extracted from four different *Azolla* species and one free-living *Anabaena azollae* isolate was examined. It was determined that the arrangement of *Anabaena azollae* and *Anabaena* 7120 *nif*HDK genes was quite similar; *nif*H and *nif*D were linked; and *nif*K was some distance away from *nif*D. It was also demonstrated that the arrangement of the *nif*HDK genes was strongly conserved among *Azolla* species, regardless of their geographical origin. Southern blots of four different *Azolla* species hybridized with the *nif*H probe from *Anabaena* 7120 are shown in Figure 15. It is important to note that slight differences in hybridization banding patterns make it possible to use DNA hybridization techniques for differentiating among cyanobacterial isolates from different *Azolla* species, even though the general arrangement of the *nif* genes was conserved.

TRANSCRIPT LEVELS OF KEY ENZYMES IN *ANABAENA AZOLLAE*

The second molecular study on *Anabaena azollae* was concerned with the quantification of transcript levels of enzymes involved either in N_2 fixation or in the heterocyst differentiation process.

Using cloned genes from *Anabaena* 7120 for GS, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), nitrogenase, and the 32-kDa protein of photosystem II, mRNA levels of the corresponding genes in the *Anabaena* endosymbiont were determined by Northern blot and dot blot hybridizations.[86]

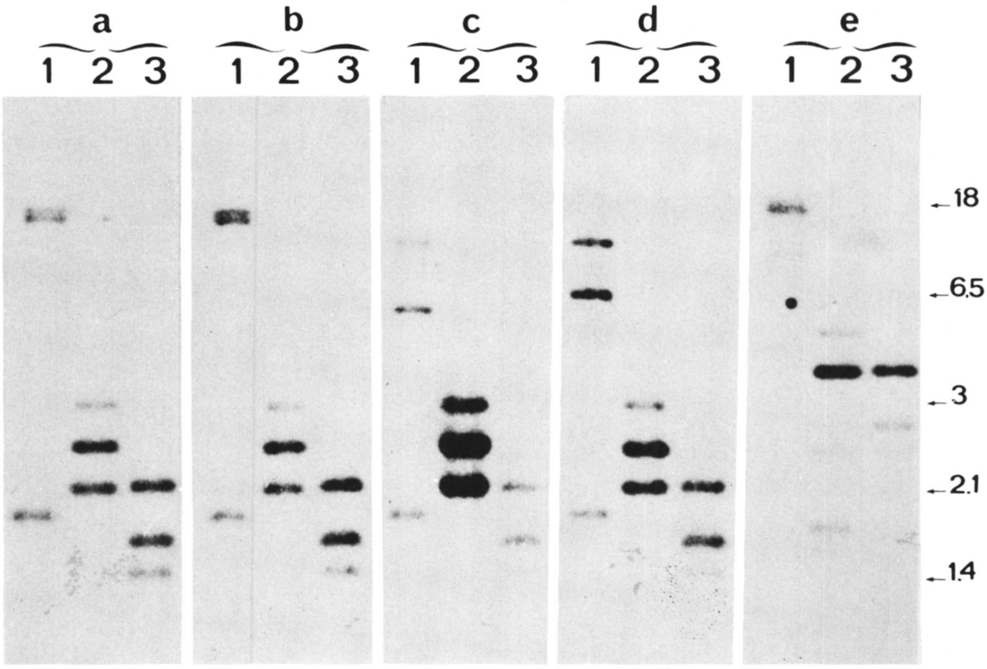


FIGURE 15. Autoradiogram showing the conservation of *nif* gene arrangement in *Anabaena azollae* associated with different *Azolla* spp. Cyanobacterial DNA was isolated from (a) *A. caroliniana*, (b) *A. filiculoides*, (c) *A. microphylla*, (d) *A. mexicana*, and (e) free-living *Anabaena azollae* originally isolated from *A. filiculoides*. Southern blots were prepared from DNA digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), and *Hin*dIII-*Eco*RI (lane 3), and hybridized with a ^{32}P-labeled *nif*H probe from *Anabaena* 7120. Sizes of DNA fragments run as standards are shown on the right. (From Franche, C. and Cohen-Bazire, G., *Plant Sci.*, 39, 125, 1985. With permission of Elsevier Scientific Publishers Ireland, Ltd.)

Glutamine Synthetase

The *Anabaena glnA* gene was of particular interest because GS is very important in the *Azolla-Anabaena* association. As discussed previously, GS activity is very low in symbiotically associated *Anabaena*, as is the level of *Anabaena* GS antigen.[134] While a vast amount of information on the regulation of GS activity in a number of different systems was available (for a review, see Stewart[140]), comparatively little was known about the regulation of GS synthesis in endosymbiotic *Anabaena*. It was reported that GS protein levels in the *Anabaena* symbiont were only 5 to 10% of those present in the free-living form.[134] On the other hand, Peters et al.[102] suggested that regulation of GS in the association is at the level of activity rather than synthesis. In this study it was shown that the GS mRNA transcript levels were tenfold lower in symbiotic vs. free-living *Anabaena* (Figure 16).[86] This result suggests that the host fern regulates transcription of the *Anabaena glnA* gene. This, of course, makes sense because the fern is prepared to utilize the ammonia given up by *Anabaena*.

In enteric bacteria, *gln*A transcription is regulated by the products of two genes, *ntr*A and *ntr*C, that respond to the ratio of glutamine to alpha-ketoglutarate. A high ratio leads to a repression of *gln*A.[141] A different mechanism would appear to be called for in the *Azolla-Anabaena* association. It would be futile for the fern to pump glutamine into the endosymbiont to shut down *gln*A because the glutamine would simply be metabolized.[134] We are currently sequencing the promoter(s) of the *gln*A gene and examining expression of *gln*A in the symbiont. This will lay the groundwork for molecular analysis of the mechanism involved in regulation of *Anabaena gln*A transcription.

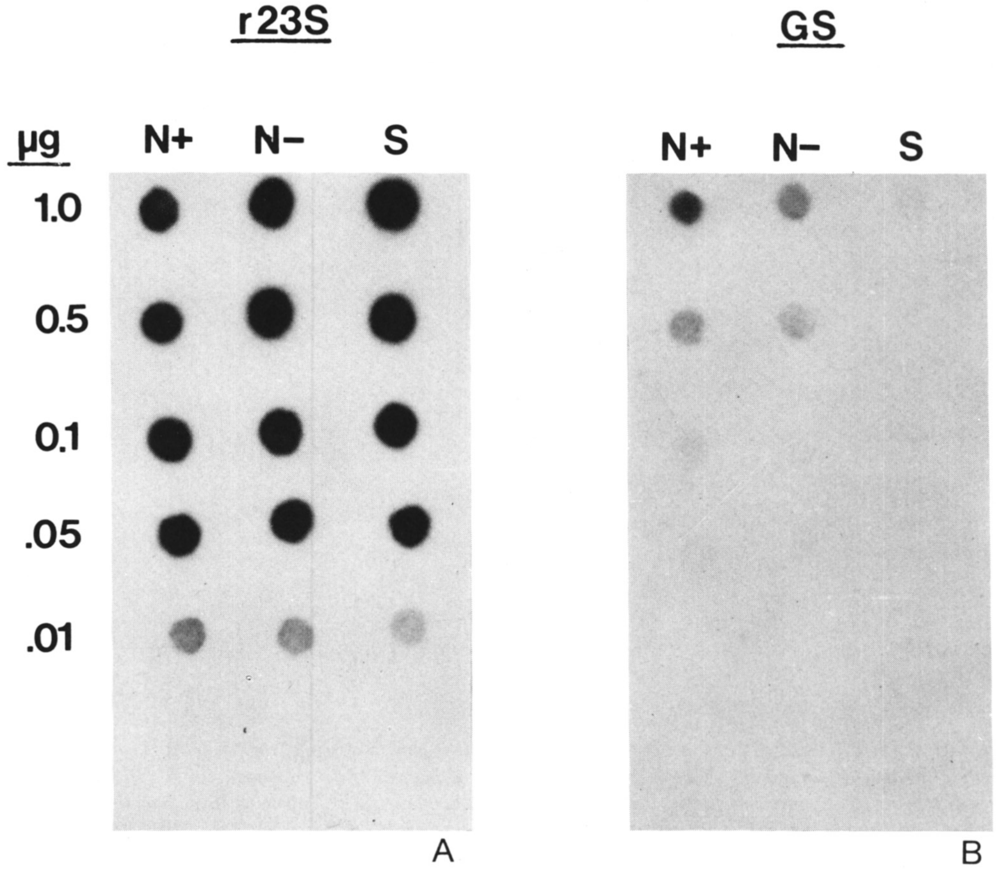


FIGURE 16. The *nif*H gene of the endosymbiont is transcribed from a fused *nif*HDK operon. A sample of 15 μg of endosymbiont RNA was denatured, fractionated on an agarose gel, and transferred to a nitrocellulose filter. The blot was hybridized with ^{32}P-labeled *nif*H DNA. Three *nif*H transcripts, 4.9, 3.0, and 1.2 kb in size, can be identified. The 4.9-kb transcript corresponds to the complete *nif*HDK operon. (From Nierzwicki-Bauer, S. A. and Haselkorn, R., *EMBO J.*, 5(1), 29, 1986. With permission of IRL Press Ltd., Oxford)

32-kDa Protein

The 32-kDa protein, which is encoded by the *psb*A gene, is required for PS II quinone-mediated electron transfer. This protein is made and rapidly turned over in unicellular cyanobacteria and in chloroplasts of higher plants.[142,143] There is only one copy of the *psb*A gene in plant chloroplast DNA, whereas there are multiple copies in *Anabaena* 7120[144] and *Anabaena azollae*.[86] It is not known how many of these copies are actually expressed in vegetative cells. Also, although heterocysts reportedly lack PS II reaction centers,[145] it is not known whether they also lack the 32-kDa protein.

In the endosymbiont, 32-kDa-specific transcripts were approximately seven to ten times more abundant than in free-living *Anabaena*[86] (Figure 17). The fact that *psb*A expression was so high in the endosymbiont cannot be explained by variations in cell populations (i.e., percentages of heterocysts and vegetative cells). While the only reported function for the 32-kDa protein is its role in PS II quinone-mediated electron transfer,[146,147] it seems likely (given the high transcript levels) that this protein has additional functions in the endosymbiont. Consequently, it is now of considerable interest to learn more about the expression and regulation of transcription of the *psb*A genes. We are currently determining whether different *psb*A copies are selectively expressed under different environmental conditions and whether the 32-kDa protein is present in heterocysts even though they lack an active PS II.

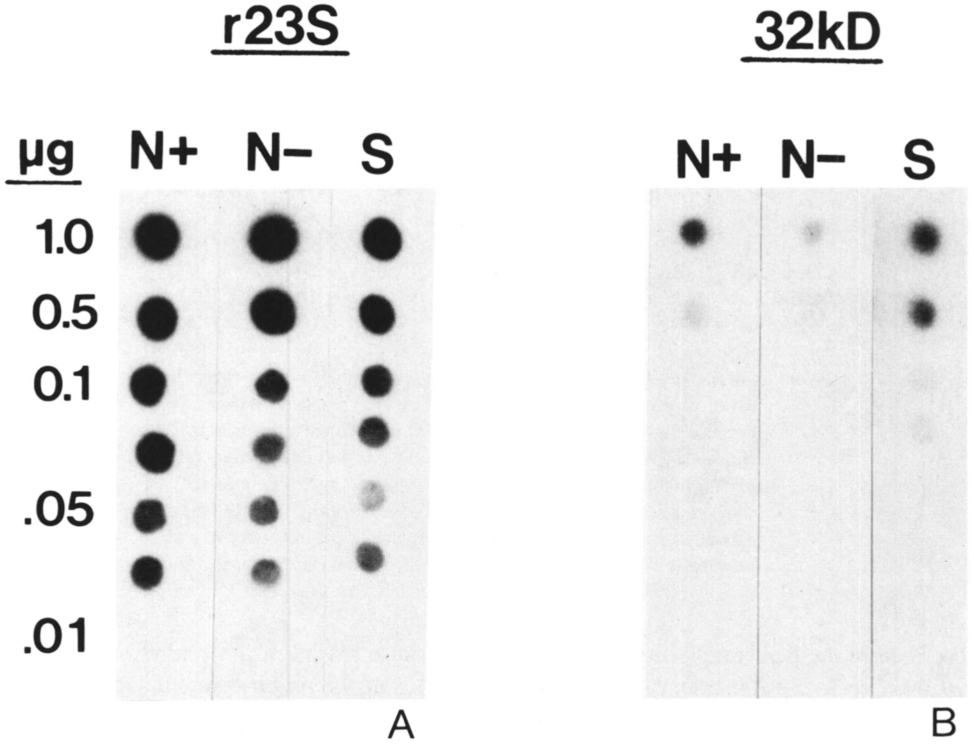


FIGURE 17. Quantitative analysis of *glnA*-specific transcripts using RNA dot blot hybridization, by hybridization of the blot with ^{32}P-labeled 23S rDNA from *Anabaena* 7120. (A) Normalization of RNA concentrations for N+, N−, and S (endosymbiont) samples. Total RNA from cells was isolated and spotted for dot blot hybridization as described in Nierzwicki-Bauer and Haselkorn.[86] Equivalent RNA concentrations of all samples (ranging from 1 to 0.01 µg per dot) are indicated by equal hybridization intensities. (B) The dot blot used for normalization in (A), rehybridized with ^{32}P-labeled *glnA* DNA. Hybridizations of equal intensity are observed to 1 µg of endosymbiont RNA (S) and 0.1 µg N+ RNA. (From Nierzwicki-Bauer, S. A. and Haselkorn, R., *EMBO J.*, 5(1), 29, 1986. With permission of IRL Press Ltd., Oxford.)

RuBP Carboxylase (RuBisCO)

Transcript levels for RuBisCO in endosymbiotic and free-living *Anabaena* were also determined.[86] RuBisCO is the first enzyme required in the Calvin pathway for the fixation of atmospheric CO_2. Like carboxylase of higher plants, the enzyme from *Anabaena* consists of eight large and eight small subunits. Using a carboxylase-specific probe, Northern dot blot hybridizations showed that carboxylase transcripts were approximately five to seven times less abundant in the symbiont than in free-living *Anabaena*. This very low amount of carboxylase message in the symbiont RNA is consistent with the reduced CO_2 fixation reported for the symbiont.[35] Quantitative results for all of the dot blot hybridizations are presented in Table 3.

nif Gene Transcripts

In addition to the quantitative results, it was found that the *nif*H, *nif*D, and *nif*K genes are transcribed from a single *nif*HDK operon in the endosymbiont[86] (Figure 18), as was originally determined for the free-living cyanobacterium *Anabaena* 7120.[148]

SOUTHERN BLOT HYBRIDIZATION STUDIES

Using the Southern blot hybridization technique, symbiotic and free-living *Anabaena* DNA were digested with the restriction enzyme *Hin*dIII and hybridized with the probes

TABLE 3
Transcript Levels in Free-Living and Symbiotic *Anabaena*

		mRNA level		
		Free-living		
Gene	**Product**	**N+**	**N−**	**Symbiont**
*gln*A	GS	100	90	10
*rbc*L-*rbc*S	RuBP carboxylase	50	100	10
*psb*A	32 kDa	15	10	100
*nif*H	Nitrogenase reductase	0	(100)	100

Note: mRNA levels are expressed relative to the highest concentration for a given probe set at 100. Comparisons cannot be made between genes because the probes were nick-translated to different specific activities. *Nif* mRNA from free-living *Anabaena azollae* was degraded to varying extents in different RNA preparations, as judged by Northern gels. These samples were not used for dot blots. The level of *nif* message was only estimated from northern gels, so the figure in parentheses has a large possible error. (From Nierzwicki-Bauer, S. A. and Haselkorn, R., *EMBO J.*, 5(1), 29, 1986. With permission of IRL Press, Oxford.)

described in the preceding section.[86] The results of these hybridization studies demonstrated that the free-living *Anabaena* strain isolated from *A. caroliniana* by Newton and Herman[47] is not identical to the major endosymbiont of *A. caroliniana*. These results show that the *gln*A, *psb*A, *rbc*S, and *nif*H genes are contained on DNA fragments of different size in free-living and freshly isolated *Anabaena azollae* (Figure 19). These results are consistent with those of the aforementioned study[139] in that a free-living strain of *Anabaena azollae* was shown to be different from its symbiotic counterpart.

CONCLUSIONS/FUTURE RESEARCH

Great progress has been made in recent years in the field of *Azolla* research. Due to advances made in the areas of (1) sexual hybridizations/crosses, (2) interchange of *Anabaena* partners, and (3) molecular studies of the *Azolla-Anabaena* association, a new era for *Azolla* research has recently begun.

At this time, it is important for research efforts to take two directions. One direction is to continue basic studies on *Azolla-Anabaena* interactions. Notably, studies should be conducted on (1) recognition processes (or lack thereof) between *Anabaena* and *Azolla*, (2) the characterization of the leaf cavity milieu, (3) control of heterocyst and akinete differentiation, and (4) the physiological capabilities of *Anabaena* present in cavities of different age. Characterization of the endosymbiotic bacteria that are found in *Azolla* leaf cavities should also continue, because these bacteria may play a significant role in the symbiosis.

The second area in which research should be concentrated is the examination of applied questions directed towards increasing the usefulness of the *Azolla-Anabaena* association as a biofertilizer. This applied research should include studies on (1) factors that induce sporulation and germination, (2) production of *Azolla* hybrids, and (3) selection of genetically superior *Azolla-Anabaena* strains.

It is only through a combination of basic and applied research that a more complete understanding of the *Azolla-Anabaena* symbiosis will be obtained. This information should make it possible to increase the nitrogen-fixing capabilities of this association through strain improvements, perhaps via genetic manipulations.

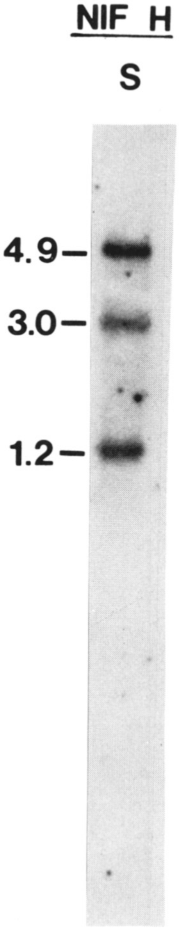


FIGURE 18. Quantitative analysis of *psb*A-specific transcripts using RNA dot blot hybridization. RNA preparations and procedures were the same as those shown in Figure 17, except that the probe was for the *psb*A gene. *Psb*A transcript levels are five to ten times more abundant in the endosymbiont RNA (lane 3) than in the N+ (lane 1) or the N− (lane 2) RNA. (From Nierzwicki-Bauer, S. A. and Haselkorn, R., *EMBO J.*, 5(1), 29, 1986. With permission of IRL Press Ltd., Oxford.)

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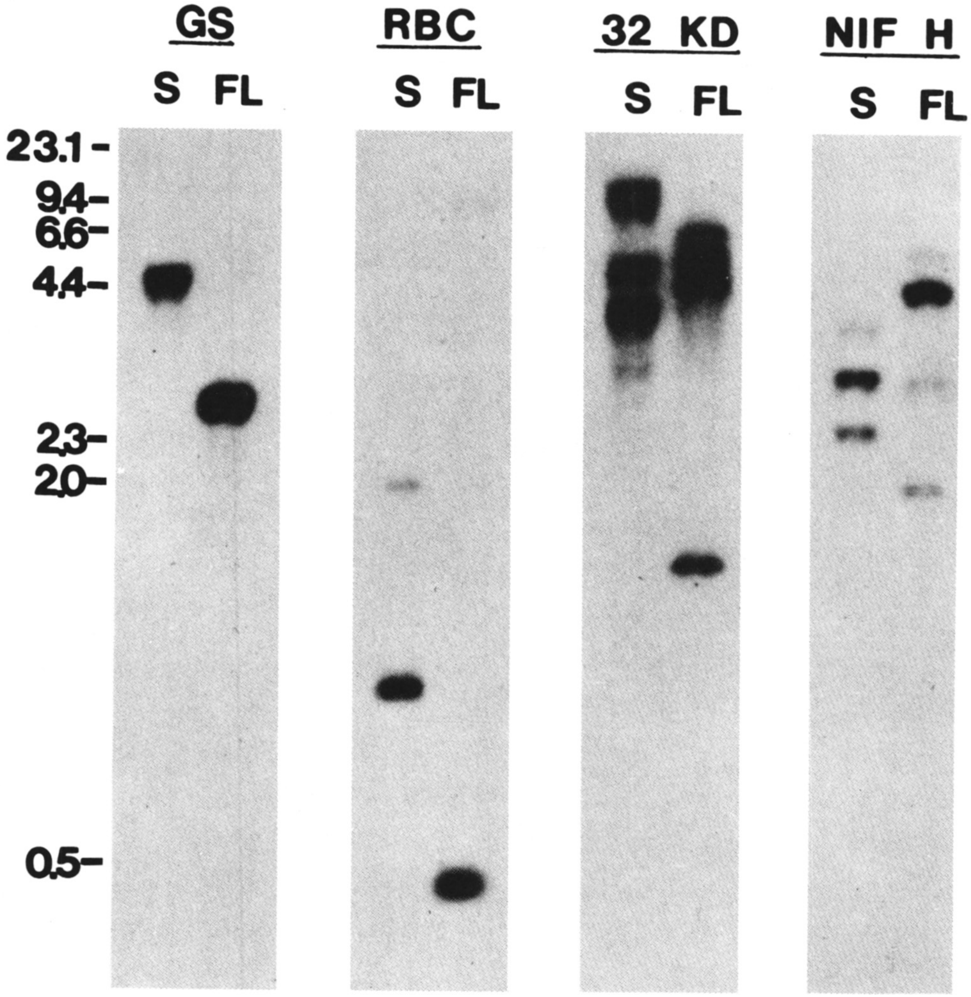

FIGURE 19. Southern hybridization of *Anabaena* 7120 gene-specific probes to total DNA from free-living *Anabaena azollae* (FL) or freshly isolated endosymbiont (S) from *A. caroliniana*. The DNA in each case was digested with *Hin*dIII. Sizes of the DNA fragments run as standards are shown in kbp on the left. Probes are described in text. Although the free-living and symbiotic *Anabaena* are clearly different organisms, the heterologous *Anabaena* 7120 probes recognize their cognate genes with equal efficiency. (From Nierzwicki-Bauer, S. A. and Haselkorn, R., *EMBO J.*, 5(1), 29, 1986. With permission from IRL Press Ltd., Oxford.)

REFERENCES

1. **Lumpkin, T. A. and Plucknett, D. L.,** Botany and ecology, in *Azolla as a Green Manure: Use and Management in Crop Production,* Westview Press, Boulder, CO, 1982, 5.
2. **Loyal, D. S., Gollen, A. K., and Ratra, R.,** Morphological and cytotaxonomic observations on *Azolla pinnata, Fern Gaz.,* 12(4), 230, 1982.
3. **Pieterse, A. H., De Lange, L., and Van Vliet, J. P.,** A comparative study of *Azolla* in the Netherlands, *Acta Bot. Neerl.,* 26(6), 433, 1977.
4. **Lumpkin, T. A.,** An introduction to *Azolla nilotica, Acta Bot. Sin.,* 23(6), 492, 1981.

5. **Peters, G. A., Toia, R. E., Jr., Evans, W. R., Crist, D. R., Mayne, B. C., and Poole, R. E.**, Characterization and comparisons of five N_2-fixing *Azolla-Anabaena* associations. I. Optimization of growth conditions for biomass increase and N content in a controlled environment, *Plant Cell Environ.*, 3, 261, 1980.
6. **Konar, R. N. and Kapoor, R. K.**, Anatomical studies on *Azolla pinnata, Phytomorphology*, 22(3,4), 211, 1972.
7. **Peters, G. A., Toia, R. E., Jr., Raveed, D., and Levine, N. J.**, The *Azolla-Anabaena azollae* relationship. VI. Morphological aspects of the association, *New Phytol.*, 80, 583, 1978.
8. **Gunning, B. E. S., Hughes, J. E., and Hardham, A. R.**, Formative and proliferative cell divisions, cell differentiation and developmental changes in the meristem of *Azolla* roots, *Planta*, 143, 121, 1948.
9. **Nierzwicki-Bauer, S. A., Balkwill, D. L., and Stevens, S. E., Jr.**, A new method for identification of heterocysts and proheterocysts in morphologically complex cyanobacteria, *Stain Technol.*, 59(3), 163, 1984.
10. **Walmsley, R. D., Breen, C. M., and Kyle, E.**, Aspects of the fern-algal relationship in *Azolla filiculoides, Limnol. Soc. S. Afr.*, 20, 13, 1973.
11. **Duckett, J. G., Toth, R., and Soni, S. L.**, An ultrastructural study of the *Azolla, Anabaena azollae* relationship, *New Phytol.*, 75, 111, 1975.
12. **Ashton, P. J. and Walmsley, R. D.**, The aquatic fern *Azolla* and its *Anabaena* symbiont, *Endeavour*, 35, 39, 1976.
13. **Uheda, E.**, Isolation of empty packets from *Anabaena*-free *Azolla, Plant Cell Physiol.*, 27(6), 1187, 1986.
14. **Tel-Or, E., Sandovsky, T., Kobiler, D., Arad, C., and Weinberg, R.**, The unique symbiotic properties of *Anabaena* in the water fern *Azolla*, in *Photosynthetic Prokaryotes: Cell Differentiation and Function*, Papageorgiou, G. C. and Packer, L., Eds., Elsevier Science, New York, 1983, 303.
15. **Newton, J. W. and Cavins, J. F.**, Altered nitrogenous pools induced by the *Azolla-Anabaena azollae* symbiosis, *Plant Physiol.*, 58, 798, 1976.
16. **Moore, A. W.**, *Azolla*: biological and agronomical significance, *Bot. Rev.*, 35, 17, 1969.
17. **Robins, R. J., Hall, D. O., Shi, D.-J., Turner, R. J., and Rhodes, M. J. C.**, Mucilage acts to adhere cyanobacteria and cultured plant cells to biological and inert surfaces, *FEMS Microbiol. Lett.*, 34, 155, 1986.
18. **Gates, J. E., Fisher, R. W., and Candler, R. A.**, The occurrence of coryneform bacteria in the leaf cavity of *Azolla, Arch. Microbiol.*, 127, 163, 1980.
19. **Calvert, H. E. and Peters, G. A.**, The *Azolla-Anabaena* relationship. IX. Morphological analysis of leaf cavity hair populations, *New Phytol.*, 89, 327, 1981.
20. **Peters, G. A., Calvert, H. E., Kaplan, D., Ito, O., and Toia, R. E., Jr.**, The *Azolla-Anabaena* symbiosis: morphology, physiology and use, *Isr. J. Bot.*, 31, 305, 1982.
21. **Calvert, H. E., Pence, M. K., and Peters, G. A.**, Ultrastructural ontogeny of leaf cavity trichomes in *Azolla* implies a functional role in metabolic exchange, *Protoplasma*, 129, 10, 1985.
22. **Peters, G. A. and Calvert, H. E.**, The *Azolla-Anabaena* symbiosis, in *Algal Symbiosis*, Goff, L. J., Ed., Cambridge University Press, Cambridge, 1983, 109.
23. **Gunning, B. E. S. and Pate, J. S.**, "Transfer cells." Plant cells with wall ingrowths specialized in relation to short distance transport of solutes — their occurrence, structure, and development, *Protoplasma*, 68, 107, 1969.
24. **Pate, J. S. and Gunning, B. E. S.**, Transfer cells, *Annu. Rev. Plant Physiol.*, 23, 173, 1972.
25. **Lang, N. J.**, Electron microscopic study of heterocyst development in *Anabaena azollae* Strasburger, *J. Phycol.*, 1, 127, 1965.
26. **Hill, D. J.**, The role of *Anabaena* in the *Azolla-Anabaena* symbiosis, *New Phytol.*, 78, 611, 1977.
27. **Neumueller, M. and Bergman, B.**, The ultrastructure of *Anabaena azollae* in *Azolla pinnata, Physiol. Plant.*, 51, 69, 1981.
28. **Grilli, M.**, Infrastrutture di *Anabaena azollae* vivente nelle foglioline di *Azolla caroliniana, Ann. Microbiol. Enzimol.*, 14, 69, 1964 (in Italian).
29. **Hill, D. J.**, The pattern of development of *Anabaena* in the *Azolla-Anabaena* symbiosis, *Planta*, 122, 179, 1975.
30. **Nierzwicki-Bauer, S. A., Balkwill, D. L., and Stevens, S. E., Jr.**, Morphology and ultrastructure of the cyanobacterium *Mastigocladus laminosus* growing under nitrogen-fixing conditions, *Arch. Microbiol.*, 137, 97, 1984.
31. **Becking, J. H.**, Ecology and physiological adaptations of *Anabaena* in the *Azolla-Anabaena* symbiosis, *Ecol. Bull. (Stockholm)*, 26, 266, 1978.
32. **Kaplan, D., Calvert, H. E., and Peters, G. A.**, The *Azolla-Anabaena azollae* relationship. XII. Nitrogenase activity and phycobiliproteins of the endophyte as a function of leaf age, *Plant Physiol.*, 80, 884, 1986.
33. **Patra, R. N. and Singh, P. K.**, Symbiotic algal heterocyst frequency in three species of *Azolla, Proc. Indian Natl. Sci. Acad.*, B50(1), 128, 1984.

34. **Wilcox, M., Mitchison, G. J., and Smith, R. J.,** Spatial control of differentiation in the blue-green alga *Anabaena*, in *Microbiology*, Schlessinger, D., Ed., American Society for Microbiology, Washington, D.C., 1975, 453.
35. **Peters, G. A.,** The *Azolla-Anabaena azollae* relationship. III. Studies on metabolic capabilities and a further characterization of the symbiont, *Arch. Microbiol.*, 103, 113, 1975.
36. **Clark, R. L. and Jensen, T. E.,** Ultrastructure of akinete development in a blue-green alga, *Cylindrospermum sp.*, *Cytologia*, 34, 439, 1969.
37. **Miller, M. M. and Lang, N. J.,** The fine structure of akinete formation and germination in *Cylindrospermum*, *Arch. Mikrobiol.*, 60, 303, 1968.
38. **Wildon, D. C. and Mercer, F. V.,** The ultrastructure of the heterocyst and akinete of the blue-green algae, *Arch. Mikrobiol.*, 47, 19, 1963.
39. **Leak, L. V. and Wilson, G. B.,** Electron microscopic observations on a blue-green alga, *Anabaena* sp., *Can. J. Genet. Cytol.*, 7, 237, 1965.
40. **Paerl, H. W.,** Specific associations of the blue-green algae *Anabaena* and *Aphanizomemon* with bacteria in fresh water blooms, *J. Phycol.*, 12, 431, 1976.
41. **Paerl, H. W. and Kellar, P. E.,** Significance of bacterial-*Anabaena* (cyanophyceae) associations with respect to nitrogen fixation in fresh water, *J. Phycol.*, 14, 254, 1978.
42. **Wiebe, W. J. and Smith, D. F.,** Direct measurement of DOC release by phytoplankton and incorporation by microheterotrophs, *Mar. Biol.*, 42, 213, 1977.
43. **Vogal, S. L., Fritch, H. L., and Gotham, I. J.,** Qualitative assay of dissolved amino acids and sugars excreted by *Chlamydomonas reinhardtii* (Chlorophyceae) and *Euglena gracilis* (Euglenophyceae), *J. Phycol.*, 14, 403, 1978.
44. **Kaplan, D. and Peters, G. A.,** The *Azolla-Anabaena azollae* relationship. X. $^{15}N_2$ fixation and transport in main stem axes, *New Phytol.*, 89, 337, 1981.
45. **Peters, G. A., Kaplan, D., Meeks, J. C., Buzby, K. M., Marsh, B. H., and Corbin, J. L.,** Aspects of nitrogen and carbon interchange in the *Azolla-Anabaena* symbiosis, in *Nitrogen Fixation and CO_2 Metabolism*, Ludden, P. W. and Burris, J. E., Eds., Elsevier Science, New York, 1985, 213.
46. **Peters, G. A. and Mayne, B. C.,** The *Azolla, Anabaena azollae* relationship. II. Localization of nitrogenase activity as assayed by acetylene reduction, *Plant Physiol.*, 53, 820, 1974.
47. **Newton, J. W. and Herman, A. I.,** Isolation of cyanobacteria from the aquatic fern, *Azolla, Arch. Microbiol.*, 120, 161, 1979.
48. **Wallace, W. H. and Gates, J. E.,** Identification of Eubacteria isolated from leaf cavities of four species of the N-fixing *Azolla* fern as *Arthrobacter* Conn and Dimmick, *Appl. Environ. Microbiol.*, 52(3), 425, 1986.
49. **Petro, M. J. and Gates, J. E.,** Distribution of *Arthrobacter* species in the leaf cavities of four species of the N-fixing *Azolla* fern, *Symbiosis*, 3, 41, 1987.
50. **Singh, P. K.,** Use of *Azolla* in rice production in India, in *Nitrogen and Rice*, International Rice Research Institute, Los Baños, Philippines, 1979, 407.
51. **Kuentzel, L. E.,** Bacteria, carbon dioxide and algal blooms, *J. Water Pollut. Control Fed.*, 41, 1737, 1969.
52. **Lange, W.,** Effect of carbohydrates in the symbiotic growth of planktonic blue-green algae with bacteria, *Nature (London)*, 215, 1277, 1967.
53. **Tison, D. L. and Lingg, A. J.,** Dissolved organic carbon utilization and O_2 uptake in algal-bacterial microcosms, *Can. J. Microbiol.*, 25, 1315, 1979.
54. **Watanabe, I.,** *Azolla-Anabaena* symbiosis — its physiology and use in tropical agriculture, in *Microbiology of Tropical Soils and Plant Productivity*, Dommergues, Y. R. and Diems, H. G., Eds., Martinus Nijhoff, The Hague, 1982, 169.
55. **Rao, H. S.,** The structure and life history of *Azolla pinnata* R. Brown with remarks on the fossil history of the Hydropterideae, *Proc. Indian Acad. Sci.*, 2, 175, 1936.
56. **Do Van Cat,** Studies on the Sexual Reproduction and Ability to Hybridize Between Species of *Azolla*, International Rice Research Institute, Los Baños, Philippines, July 1985.
57. **Singh, A. and Srivastava, O. N.,** Effect of light intensity on the growth of *Azolla pinnata* R. Brown at Ranchi, India, *Hydrobiologica*, 126, 49, 1985.
58. **Becking, J. H.,** Environmental requirements of *Azolla* for use in tropical rice production, in *Nitrogen and Rice*, International Rice Research Institute, Los Baños, Philippines, 1979, 345.
59. **Singh, A. and Srivastava, O. N.,** Effect of photoperiod on the growth of *Azolla pinnata* R. Brown, *Hydrobiologia*, 123, 211, 1985.
60. **Holst, R. W. and Yopp, J. H.,** Studies of the *Azolla-Anabaena* symbiosis using *Azolla mexicana*. I. Growth in nature and laboratory, *Am. Fern J.*, 69(1), 1979.
61. **Perkins, S. K., Peters, G. A., Lumpkin, T. A., and Calvert, H. E.,** Scanning electron microscopy of perine architecture as a taxonomic tool in the genus *Azolla* Lamarck, *Scanning Electron Microsc.*, 4, 1719, 1985.

62. **Duncan, R. E.**, The cytology of sporangium development in *Azolla filiculoides*, *Bull. Torrey Bot. Club*, 67, 391, 1940.
63. **Konar, R. N. and Kapoor, R. K.**, Embryology of *Azolla pinnata*, *Phytomorphology*, 24, 228, 1974.
64. **Demalsey, P.**, Le Sporophyte d' *Azolla nilotica*, *Cellule*, 56, 5, 1953.
65. **Calvert, H. E., Perkins, S. K., and Peters, G. A.**, Sporocarp structure in the heterosporous water fern *Azolla mexicana* Presl, *Scanning Electron Microsc.*, 3, 1499, 1983.
66. **Fowler, K. and Stenett-Willson, J.**, Sporoderm architecture in modern *Azolla*, *Fern Gaz.*, 11, 404, 1978.
67. **Lucas, R. C. and Duckett, J. G.**, A cytological study of the male and female sporocarps of the heterosporous water fern *Azolla filiculoides* Lam., *New Phytol.*, 85, 409, 1980.
68. **Martin, A. R. H.**, Some structures in *Azolla* megaspores and an anomalous form, *Rev. Palaeobot. Palynol.*, 21, 141, 1976.
69. **Herd, Y. R., Cutter, E. G., and Watanabe, I.**, An ultrastructural study of postmeiotic development in the megasporocarp of *Azolla microphylla*, *Can. J. Bot.*, 64, 822, 1986.
70. **Lumpkin, T. A. and Plucknett, D. L.**, *Azolla*: botany, physiology and use as a green manure, *Econ. Bot.*, 34(2), 111, 1980.
71. **Toia, R. E., Jr., Marsh, B. H., Perkins, S. K., McDonald, J. W., and Peters, G. A.**, Sporopollenin content of the spore apparatus of *Azolla*, *Am. Fern J.*, 75(2), 38, 1985.
72. **Fowler, K.**, Megaspores and massulae of *Azolla prisca* from the Oligocene of the Isle of Wight, *Paleontology*, 18, 483, 1975.
73. **Hills, L. V. and Gopal, B.**, *Azolla primaeva* and its phylogenetic significance, *Can. J. Bot.*, 45, 1179, 1967.
74. **Xiao, Quin-yuan, Shi, Yan-ru, Yang, Guang-ji, and Peng, Ke-lin**, Techniques for sexual reproduction of *Azolla filiculoides*, The Soil and Fertilizer Institute, Hunan Academy of Agriculture and Science, 1986.
75. **Cang Lin, I., Watanabe, I., Liu, C. C., Zheng, D. V., and Tang, L. F.**, Re-establishment of symbiosis to *Anabaena*-free *Azolla*, in *Nitrogen Fixation: Hundred Years After*, Bothe, H., de Bruijn, F. J., and Newton, W. E., Eds., Gustav Fischer, Stuttgart, 1988, 223.
76. **Liu, C. C.**, Use of *Azolla* in rice production in China, in *Nitrogen and Rice*, International Rice Research Institute, Los Baños, Philippines, 1979, 375.
77. **Lamarck, J. B. P. A. M.**, *Azolla filiculoides* Lam., *Encyl. Meth. Bot.*, 1, 343, 1783.
78. **Ashton, P. J. and Walmsley, R. D.**, The taxonomy and distribution of *Azolla* species in southern Africa, *Bot. J. Linnean Soc.*, 89, 239, 1984.
79. **Sweet, A. and Hills, L. V.**, A study of *Azolla pinnata* R. Brown, *Am. Fern J.*, 61, 1, 1971.
80. **Ladha, J. K. and Watanabe, I.**, Antigenic similarity among *Anabaena azollae* separated from different species of *Azolla*, *Biochem. Biophys. Res. Commun.*, 109(3), 675, 1982.
81. **Gates, J. E., Fisher, R. W., Goggin, T. W., and Azrolan, N. I.**, Antigenic differences between *Anabaena azollae* fresh from the *Azolla* fern leaf cavity and free-living cyanobacteria, *Arch. Microbiol.*, 128, 126, 1980.
82. **McCowen, S. M., MacArthur, L., and Gates, J. E.**, *Azolla* fern lectins that specifically recognize endosymbiotic cyanobacteria, *Curr. Microbiol.*, 14, 329, 1987.
83. **Mellor, R. B., Gadd, G. M., Rowell, P., and Stewart, W. D. P.**, A phytohaemagglutinin from the *Azolla-Anabaena* symbiosis, *Biochem. Biophys. Res. Commun.*, 99, 1348, 1981.
84. **Arad, H., Keysari, A., Tel-Or, E., and Kobiler, D.**, A comparison between cell antigens in different isolates of *Anabaena azollae*, *Symbiosis*, 1, 195, 1985.
85. **Ladha, J. K. and Watanabe, I.**, Antigenic analysis of *Anabaena azollae* and the role of lectin in the *Azolla-Anabaena* symbiosis, *New Phytol.*, 98, 295, 1984.
86. **Nierzwicki-Bauer, S. A. and Haselkorn, R.**, Differences in mRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*, *EMBO J.*, 5(1), 29, 1986.
87. **Schmidt, E. L.**, Initiation of plant root-microbe interactions, *Annu. Rev. Microbiol.*, 335, 355, 1979.
88. **Kobiler, D., Cohen-Sharon, A., and Tel-Or, E.**, Recognition between the N_2-fixing *Anabaena* and the water fern *Azolla*, *FEBS Lett.*, 133(1), 157, 1981.
89. **Kobiler, D., Cohen-Sharon, A., and Tel-Or, E.**, Lectins are involved in the recognition between *Anabaena* and *Azolla*, *Isr. J. Bot.*, 31, 324, 1982.
90. **Zimmerman, W. J.**, Biomass and pigment production in three isolates of *Azolla*. I. Response to water stress, *Ann. Bot.*, 56, 689, 1985.
91. **Brotonegoro, S. and Abdulkadir, S.**, Growth and nitrogen-fixing activity of *Azolla pinnata*, *Ann. Bogor.*, 6(2), 69, 1976.
92. **Singh, P. K.**, Multiplication and utilization of fern "*Azolla*" containing nitrogen-fixing algal symbiont as green manure in rice cultivation, *Riso*, 26, 125, 1977.
93. **Ashton, P. J.**, The effect of some environmental factors in the growth of *A. filiculoides* Lam., in *The Orange River Progress Report*, Zinderen-Bakker, E. M. Y., Ed., University of the Orange Free State, Bloemfontein, South Africa, 1974, 123.

94. **Reynaud, P. A.,** The use of *Azolla* in West Africa, in Biological Nitrogen Fixation Technology for Tropical Agriculture, Graham, P. H. and Harris, S. C., Eds., papers presented at a workshop held at Centro Internacional de Agricultura, Cali, Colombia, March 9 to 13, 1981, 565.
95. **Peters, G. A. and Calvert, H. E.,** The *Azolla-Anabaena* symbiosis, in *Advances in Agricultural Microbiology,* Subba Rao, N. S., Ed., Oxford and IBH Publishing Co., New Delhi, Bombay, 1982, 191.
96. **Yatazawa, M., Tomomatsu, N., Hosoda, N., and Nunome, K.,** Nitrogen fixation in *Azolla-Anabaena* symbiosis as affected by mineral nutrient status, *Soil Sci. Plant Nutr. (Tokyo),* 26(3), 415, 1980.
97. **Watanabe, I., Espinas, C. R., Berja, N. S., and Alimagno, B. V.,** The Utilization of the *Azolla-Anabaena* complex as a nitrogen fertilizer for rice, *Int. Rice Res. Pap. Ser.,* No. 11, 1, 1977.
98. **Olsen, C.,** On biological nitrogen fixation in nature, particularly in blue-green algae, *C.R. Trav. Lab. Carlsberg,* 37, 269, 1972.
99. **Johnson, G. V., Mayeux, P. A., and Evans, H. J.,** A cobalt requirement for symbiotic growth of *Azolla filiculoides* in the absence of combined nitrogen, *Plant Physiol.,* 41, 852, 1966.
100. **Tung, H. F. and Watanabe, I.,** Differential response of *Azolla-Anabaena* associations to high temperature and minus phosphorous treatments, *New Phytol.,* 93, 423, 1983.
101. **Talley, S. N. and Rains, D. W.,** *Azolla* as a nitrogen source for temperate rice, in *Nitrogen Fixation,* Vol. 2, Newton, W. E. and Orme-Johnson, W. H., Eds., University Park Press, Baltimore, 1980, 311.
102. **Peters, G. A., Ray, T. B., Mayne, B. C., and Toia, R. E., Jr.,** *Azolla-Anabaena* association: morphological and physiological studies, in *Nitrogen Fixation,* Vol. 2, Newton, W. E. and Orme-Johnson, W. H., Eds., University Park Press, Baltimore, 1980, 293.
103. **Talley, S. N., Talley, B. J., and Rains, D. W.,** Nitrogen fixation by *Azolla* in rice fields, in *Genetic Engineering for Nitrogen Fixation,* Hollaender, A., Ed., Plenum Press, New York, 1977, 259.
104. **Ahmad, G.,** Effect of light intensity on the growth of *Azolla filiculoides, J. Indian Bot.,* 20, 213, 1941.
105. **Talley, S. N. and Rains, D. W.,** *Azolla filiculoides* Lam. as a fallow season green manure for rice in a temperate climate, *Agron. J.,* 72, 11, 1980.
106. **Lumpkin, T. A. and Bartholomew, D. P.,** Crop physiology and metabolism. Predictive models for the growth response of eight *Azolla* accessions to climatic variables, *Crop Sci.,* 26, 107, 1986.
107. **Li, Z. X.,** *Organic Matter and Rice,* International Rice Research Institute, Los Baños, Philippines, 169, 1984.
108. **Brontonegoro, S., Sudjadi, M., Partohardjono, S., Sukiman, H., Prihatini, T., and Hendriks, V.,** Some experiments on the use of *Azolla* for rice production in Indonesia, in *Biological Nitrogen Fixation for Tropical Agriculture,* Graham, P. H. and Harris, S. C., Eds., Centro International de Agricultura Tropical, Cali, Colombia, 1982, 567.
109. **Tuan, D. T. and Thuyet, T. Q.,** Use of *Azolla* in rice production in Vietnam, in *Nitrogen and Rice,* International Rice Research Institute, Los Baños, Philippines, 1979, 395.
110. **Peters, G. A., Mayne, B. C., Ray, T. B., and Toia, R. E., Jr.,** Physiology and biochemistry of the *Azolla-Anabaena* symbiosis, in *Nitrogen and Rice,* International Rice Research Institute, Los Baños, Philippines, 1979, 325.
111. **Peters, G. A.,** The *Azolla-Anabaena azollae* symbiosis, in *Genetic Engineering for Nitrogen Fixation,* Hollaender, A., Ed., Plenum Press, New York, 1977, 231.
112. **Peters, G. A., Ito, O., Tyagi, V. V. S., and Kaplan, D.,** Physiological studies on N_2-fixing *Azolla,* in *Genetic Engineering of Symbiotic Nitrogen Fixation and Conservation of Fixed Nitrogen,* Lyons, J. M., Valentine, R. C., Phillips, D. A., Rains, D. W., and Huffaker, R. C., Eds., Plenum Press, New York, 1981, 343.
113. **Peters, G. A. and Mayne, B. C.,** The *Azolla, Anabaena azollae* relationship. I. Initial characterization of the association, *Plant Physiol.,* 53, 813, 1974.
114. **Ventkataraman, O. S.,** Studies on nitrogen fixation by blue-green algae. III. Nitrogen fixation by *Anabaena azollae, Indian J. Agric. Sci.,* 32, 22, 1962.
115. **Wieringa, K. T.,** A new method for obtaining bacteria-free cultures of blue-green algae, *Antonie van Leeuwenhoek; J. Microbiol. Sero.,* 34, 54, 1968.
116. **Peters, G. A., Toia, R. E., Jr., Calvert, H. A., and Marsh, B. H.,** Lichens to *Gunnera*-with emphasis on *Azolla, Plant Soil,* 90, 17, 1986.
117. **Ray, T. B., Mayne, B. C., Toia, R. E., Jr., and Peters, G. A.,** *Azolla-Anabaena* relationship. VIII. Photosynthetic characterization of the association and individual partners, *Plant Physiol.,* 64, 791, 1979.
118. **Haselkorn, R.,** Heterocysts, *Annu. Rev. Plant Physiol.,* 29, 319, 1978.
119. **Tinh, N. H. and Faludi-Daniel, A.,** Photosynthetic activity and ^{14}C-labelling pattern in *Azolla pinnata* at various temperatures, *Photosynthetica,* 15(1), 87, 1981.
120. **Peters, G. A., Ito, O., Tyagi, V. V. S., Mayne, B. C., Kaplan, D., and Calvert, H. E.,** Photosynthesis and N_2 fixation in the *Azolla-Anabaena* symbiosis, in *Current Perspectives in Nitrogen Fixation,* Gibson, A. H. and Newton, W. E., Eds., Australian Academy of Sciences, Canberra, 1981.
121. **Bidwell, R. G. S.,** Photosynthesis and light and dark respiration in freshwater algae, *Can. J. Bot.,* 55, 809, 1977.

122. **Lloyd, N. D. H., Canvin, D. J., and Culver, D. A.,** Photosynthesis and photorespiration in algae, *Plant Physiol.*, 59, 936, 1977.
123. **Ray, T. B., Peters, G. A., Toia, R. E., Jr., and Mayne, B. C.,** *Azolla-Anabaena* relationship. VII. Distribution of ammonia-assimilating enzymes, protein, and chlorophyll between host and symbiont, *Plant Physiol.*, 62, 463, 1978.
124. **Tyagi, V. V. S., Ray, T. B., Mayne, B. C., and Peters, G. A.,** The *Azolla-Anabaena azollae* relationship. XI. Phycobilins in the action spectrum for nitrogenase-catalyzed acetylene reduction, *Plant Physiol.*, 68, 1479, 1981.
125. **Tyagi, V. V. S., Mayne, B. C., and Peters, G. A.,** Purification and initial characterization of phycobiliproteins from the endophytic cyanobacterium of *Azolla, Arch. Microbiol.*, 128, 41, 1980.
126. **Chan-van-Ni, Chan-Hai and Gogotov, I. N.,** Photosynthesis and hydrogen metabolism in *Anabaena azollae, Mikrobiologiya,* 52(6), 896, 1983.
127. **Tang, P. S., Ding-ji, S., and Changzheng, H.,** Regulation of energy metabolism (photosynthesis and nitrogen fixation) in blue-green algae, in Proc. Joint China-U.S. Phycology Symp., November 15 to 21, 1981, 339.
128. **Holst, R. W.,** Anthocyanins of *Azolla, Am. Fern J.*, 67, 99, 1977.
129. **Becking, J. H. and Donze, M.,** Pigment distribution and nitrogen fixation in *Anabaena azollae, Plant Soil,* 61, 203, 1981.
130. **Vincenzini, M., Margheri, M. C., and Sili, C.,** Outdoor mass culture of *Azolla* spp.: yields and efficiencies of nitrogen fixation, *Plant Soil,* 86, 57, 1985.
131. **Peters, G. A., Toia, R. E., Jr., and Lough, S. M.,** *Azolla-Anabaena* relationship. V. $^{15}N_2$ fixation, acetylene reduction, and H_2 production, *Plant Physiol.*, 59, 1021, 1977.
132. **Peters, G. A., Evans, W. R., and Toia, R. E., Jr.,** *Azolla-Anabaena azollae* relationship. IV. Photosynthetically driven, nitrogenase-catalyzed H_2 production, *Plant Physiol.*, 58, 119, 1976.
133. **Corbin, J. L., Marsh, B. H., and Peters, G. A.,** *N*-γ-L-Glutamyl-β-D-aminophenylpropanoic acid, a dipeptide from the aquatic fern, *Azolla caroliniana, Phytochemistry,* 25(2), 527, 1986.
134. **Orr, J. and Haselkorn, R.,** Regulation of glutamine synthetase activity and synthesis in free-living and symbiotic *Anabaena* spp., *J. Bacteriol.*, 152(2), 626, 1982.
135. **Uheda, E.,** Isolation of hair cells from *Azolla filiculoides* var. *japonica* leaves, *Plant Cell Physiol.*, 27(7), 1255, 1986.
136. **Ito, O. and Watanabe, I.,** The relationship between combined nitrogen uptakes and nitrogen fixation in *Azolla-Anabaena* symbiosis, *New Phytol.*, 95, 647, 1983.
137. **Lee, C. C. and Lin, C. J.,** The possibility of using *Azolla* as a source of nitrogen for rice in Taiwan, *Soils Fert. Taiwan,* 35, 1981.
138. **Ohmori, M. and Hattori, A.,** Effect of ammonia on nitrogen fixation by the blue-green alga *Anabaena cylindrica, Plant Cell Physiol.*, 15, 131, 1974.
139. **Franche, C. and Cohen-Bazire, G.,** The structural *nif* genes of four symbiotic *Anabaena azollae* show a highly conserved physical arrangement, *Plant Sci.*, 39, 125, 1985.
140. **Stewart, W. D. P.,** Some aspects of structure and function in N_2-fixing cyanobacteria, *Annu. Rev. Microbiol.*, 34, 497, 1980.
141. **Haselkorn, R.,** Organization of the genes for nitrogen fixation in photosynthetic bacteria and cyanobacteria, *Annu. Rev. Microbiol.*, 40, 525, 1986.
142. **Eaglesham, A. R. J. and Ellis, R. J.,** Protein synthesis in chloroplasts. II. Light-driven synthesis of membrane proteins by isolated pea chloroplasts, *Biochim. Biophys. Acta,* 335, 396, 1974.
143. **Reisfeld, A., Matoo, A., and Edelman, M.,** Processing of a chloroplast-translated membrane protein *in vivo.* Analysis of the rapidly synthesized 32,000-dalton shield protein and its precursor in *Spirodela oligorrhiza, Eur. J. Biochem.*, 124, 125, 1982.
144. **Curtis, S. E. and Haselkorn, R.,** Isolation, sequence and expression of two members of the 32-kD thylakoid membrane protein gene family from the cyanobacterium *Anabaena* 7120, *Plant Mol. Biol.*, 3, 249, 1984.
145. **Tel-Or, E. and Stewart, W. D. P.,** Photosynthetic components and activities of nitrogen-fixing isolated heterocysts of *Anabaena cylindrica, Proc. R. Soc. London Ser. B,* 198, 61, 1977.
146. **Arntzen, C. J., Darr, S. C., Mullet, J. E., Steinback, K. E., and Pfister, K.,** Polypeptide determinants of plastoquinone function in photosystem II of chloroplasts, in *Functions of Quinones in Energy Conserving Systems,* Trumpower, B., Ed., Academic Press, New York, 1982, 443.
147. **Mazur, B. J., Rice, D. and Haselkorn, R.,** Identification of blue-green algal nitrogen-fixation genes using heterologous DNA hybridization probes, *Proc. Natl. Acad. Sci. U.S.A.*, 77, 186, 1980.
148. **Golden, J. W., Robinson, S. J., and Haselkorn, R.,** Re-arrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena, Nature (London),* 314, 419, 1985.

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Chapter 5

AZOLLA-ANABAENA SYMBIOSIS: USE IN AGRICULTURE

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INTRODUCTION

Azolla is a fast-growing floating aquatic fern which lives in symbiotic association with the filamentous nitrogen-fixing cyanobacterium *Anabaena*. The *Azolla-Anabaena* association has a tremendous potential for providing photosynthetic production of nitrogen fertilizer. This symbiotic association has been used as a green manure in rice fields in Vietnam and southern China for centuries.[1,2] For a historical perspective on the use of *Azolla* in Vietnam and China, the reader is referred to Tuan and Thuyet[2] and Liu.[3]

Although *Azolla-Anabaena* has been used for hundreds of years, it was not until the late 1950s that techniques for growing *Azolla* as a nitrogen fertilizer for rice were extensively studied. In the past 25 to 30 years, a tremendous amount has been learned about this symbiosis and techniques for cultivating and using it as an alternative to chemical nitrogen fertilizers. *Azolla* is currently used in association with rice cultures in Thailand,[4,5] Indonesia,[6,7] India,[8,9] People's Republic of China,[3,10] Philippines,[11] Vietnam,[2,12] and Taiwan.[13,14] *Azolla* is most extensively used in the People's Republic of China, which is very advanced in practical applications of *Azolla* for agricultural production.[10] *Azolla* is one of the most important green manures in China, where currently there are more than 1.34 million hectares of rice fields cultivated with *Azolla*.[3] *Azolla* has also been used as a green manure in Brazil,[15] Italy,[16] Mexico,[17] and West Africa.[18]

The purpose of this chapter is to review and summarize information that is currently available on the agricultural applications of *Azolla*. Major topics which have been included are utilization of *Azolla* as an alternative nitrogen fertilizer, additional uses of Azolla, optimization of growth and nitrogen-fixing capabilities of *Azolla*, and *Azolla* pests and pest control. The chapter concludes with suggested future research for improved utilization of the *Azolla-Anabaena* symbiosis.

AZOLLA AS A NITROGEN SOURCE

Agronomically significant N_2 fixation has been defined as that higher than 5 kg N per hectare per one crop of rice.[19,20] Based on this definition, there is no question that *Azolla* has a high biological potential as a green manure with significant N_2-fixing capabilities. In fact, the annual N_2 gain by *Azolla* has been reported to be equivalent to that of forage leguminous crops.[21] Thus, the question is: to what extent can *Azolla* N_2 fixation replace requirements for chemical nitrogen fertilizers? To answer this question, a number of factors must be considered.

First, it is necessary to determine the maximum annual nitrogen gain that could be achieved through growth and incorporation of *Azolla* in different geographical locations under ideal growth conditions. While a number of estimates of *Azolla* nitrogen input have been made, these estimates vary considerably because they are influenced by numerous factors. These include the agricultural system used, fertilization requirements of the field, the presence of soluble iron, problems with pests, etc. Maximum estimated inputs during exponential growth of *Azolla* assuming unlimited growth range from 335 to 670 kg N per hectare per year.[7,22,23] Adjusted values, taking into account that (1) *Azolla* growth is actually discontinuous and (2) there is dark nitrogen fixation occurring at a rate of 20 to 30%, range

TABLE 1
N_2 Gain Achieved Using *Azolla*

Species	Amount of N	Location	Ref.
Azolla pinnata	335—670 kg/ha/year	Indonesia	24
A. pinnata	465 kg/ha/year (in total of 22 crops)	Philippines	21
A. pinnata	330 kg/ha/220 d	Philippines	11
A. pinnata	840 kg/total/year	India	9
A. pinnata (Da Li Clone)	80 kg/ha/year (in 2 seasons)	Taiwan	13
A. filiculoides	500 kg/ha/year	Sacramento Valley	25
A. filiculoides	145 kg/ha/6 months (fallow season)	North America	26
A. caroliniana	380 kg/ha/year	Philippines	27
A. caroliniana	103—162 kg/ha/year	Indonesia	7

TABLE 2
Range of Average Nitrogen-Fixing Rates for Different *Azolla* Species

Species	Average nitrogen-fixing rates (kg N/ha/d)	Ref.
Azolla filiculoides	2.5	26, 28
A. filiculoides	1.5—2.6	29
A. mexicana	1.0	25[a]
A. caroliniana	3.6	27[a]
A. pinnata	0.4—3.4	29
A. pinnata	1.1	11
A. pinnata	1.4	21

Note: Adapted from Kikuchi et al. 1985.[29]

[a] Only one reported.

TABLE 3
Grain Yield Increases due to Incorporation of *Azolla* Nitrogen

Maximum grain yield increases	Ref.
14—40% (from different studies)	4
25%	11
9—38%	8
18.6%	3
38—40%	18

from 103 to 162 kg N per hectare per year.[23] Estimated values for different geographical areas and different *Azolla* species are listed in Table 1. Average nitrogen-fixation rates (kilograms N per hectare per day) for various *Azolla* species are listed in Table 2.

Cultivation of *Azolla* not only increased grain and straw yield, but also improved the quality of grain by increasing its protein content.[3,8] *Azolla* incorporation caused rice to grow better and flower earlier.[9] Increases in grain yield due to the incorporation of *Azolla* nitrogen ranged from 14 to 40%. Specific grain yield increases obtained in different studies are recorded in Table 3. ^{15}N studies demonstrated that of the *Azolla* nitrogen used by rice, 45 to 50% was incorporated into rice straw, 30 to 45% into rice grain, and 10 to 20% into rice roots.[30] This distribution was the same regardless of whether *Azolla* was placed on the soil surface, grown on water, or incorporated into the soil.[30]

TABLE 4
Suggested Inoculum Density of *Azolla*

Fresh weight (kg/m^2)	Purpose	Location	Ref.
0.001—0.01	Basal green manure or dual-cropping	Philippines, S. Cotabato	29
0.40	Basal green manure; covers area in 12 days	Indonesia, West Java	6
0.25—0.50	Basal green manure	Vietnam	2
0.50—0.75	Basal green manure	Vietnam	12
0.25—0.50	Dual-cropping	Vietnam	12
0.50—0.75	Multiplication beds	Vietnam	2, 12
0.50	Half-saturation technique	Vietnam	31
0.75—1.0	Production of animal feed	Vietnam	2
0.075	Basal green manure	Taiwan	14
0.05	Dual-cropping	Taiwan	14
0.50	Dual-cropping	China	10
0.50—0.75	Dual-cropping; covers area in 10—15 days	China	3
0.20	Basal green manure; covers area in 10—20 days	India	9
0.10	Dual-cropping	India	9
0.02—0.10	Dual-cropping: rain-fed areas; covers area in 20—40 days	India	9
0.10—0.40	Multiplication beds	India	9
0.05	Fallow season green manure; covers area in 15 days	North America	26

Numerous studies have indicated that growth of *Azolla* before or after rice planting is equivalent to the application of 30 to 40 kg N fertilizer per hectare in the form of urea or ammonium sulfate.[9,28,29,31] Thus, approximately 5 to 8 tons of *Azolla* per hectare gives an increase in grain yield equal to 30 kg N fertilizer per hectare.[32] Growth of *Azolla* and incorporation before and after the rice crop was reported to be equivalent to 60 to 100 kg N fertilizer per hectare.[14,28,31] Thus, nitrogen efficiency of *Azolla* is almost comparable to[9,11] or slightly less than that of urea or ammonium sulfate.[11,28,33] However, it has been suggested that *Azolla* is less efficient in supplying nitrogen to rice due to the time required for its decomposition and release of fixed nitrogen.[33]

An alternative to using either *Azolla* or chemical nitrogen fertilizers is to use a combination of the two. Studies have reported that paddies fertilized with *Azolla* in combination with urea gave greater yields than those fertilized with urea alone.[6] It was also found that ammonium sulfate and ammonium phosphate could effectively be used in dual-culture of *Azolla* and rice.[13] Ammonium nitrate and chloride increased *Azolla* growth, while ammonium acetate, carbonate, and citrate decreased growth.[13] As much as 25 kg of chemical N fertilizer per hectare could be used without inhibiting heterocyst differentiation in the *Azolla-Anabaena* association.[8]

AZOLLA INOCULA
Inoculum Density

When using *Azolla* as a nitrogen fertilizer, it is important to determine the appropriate inoculum density for efficient growth/production. If the inoculum density is too low, *Azolla* can be overgrown by algae and weeds.[31] Conversely, if the inoculum density is too high, both growth rates and nitrogen fixation rates are reduced.[34]

Regardless of the inoculum size, the *Azolla* inoculum should always be fresh and healthy. The exact inoculum density is dependent upon specific growth conditions and therefore differs in various geographical locations. Also, the inoculum density is different when *Azolla* is grown for different purposes. The suggested inocula for different geographical locations and purposes are listed in Table 4.

Propagation of *Azolla* Inocula

Azolla inocula are continuously grown in so-called multiplication centers or preparation plots and ponds. Multiplication centers are very common in Vietnam, where *Azolla* is multiplied throughout the summer. In August, *Azolla* inocula are provided to the farmers.[2,31] The inocula are then further multiplied in the rice fields. Specific inoculation techniques for *Azolla* are (1) hand carrying and spreading (broadcasting), (2) the half-saturation technique, and (3) use of water currents along irrigation canals.[29]

The half-saturation technique is a means of exponentially expanding *Azolla* propagation while distributing it into rice fields.[12,31] The basics of this technique follow. Initially, *Azolla* is introduced into one area at an inoculum density of 0.5 kg fresh weight *Azolla* per square meter. Once *Azolla* has fully covered the growth area (approximately 1 week), half the *Azolla* is transferred to a new area of similar size. When this area is completely covered, then half the *Azolla* is used to inoculate a third area, and so forth.

Alternatively, *Azolla* inocula may be propagated on fallow land.[3,9] As a general rule, if 5 to 10% of the land is used to cultivate *Azolla*, then enough *Azolla* will be produced to fertilize the remaining land in 2 to 3 months.[9] In India, fallow land used for *Azolla* propagation is often a reserved area for rice crops on cooperative farms.

Potential Use of Spores as Inocula

While *Azolla* can reproduce sexually via sporocarp formation, it is not yet practical to use fertilized megaspores as the inoculum for rice paddies for the following reasons. First, the environmental factors responsible for triggering sporocarp formation are not well understood. It is only known that temperature, light quality and quantity, photoperiod, and plant density are somehow involved with triggering sporulation.[31] Thus, it is not possible to intentionally induce sporocarp formation in order to produce large amounts of spores for storage and seeding. Second, germinating megaspores grow very slowly (which may restrict using spores as inocula).[31] The possibility, however, that sporocarps could be used for effective oversummering and inocula necessitates additional research on *Azolla* sporocarps and the sporulation process. For a more detailed discussion of the *Azolla* sporulation process, the reader is referred to the preceding chapter.

Storage and Propagation of *Azolla*

Overwintering, spring nursery propagation, oversummering, and autumn nursery propagation are all techniques used to store or propagate *Azolla* inocula throughout the year. Overwintering techniques are used to keep *Azolla* warm during the winter; conversely, oversummering techniques are used to keep *Azolla* cool during the summer. Spring and autumn propagation provides large quantities of *Azolla* for use as inocula.

Overwintering

There are three commonly used overwintering techniques, all of which involve artificial methods of keeping *Azolla* warm. In the northern provinces of China, covered nurseries are used most often for overwintering.[3,10] *Azolla* is moved into nursery beds in November and maintained at a density of 0.75 to 1.0 kg per square meter. One type of overwintering nursery that is used in China is shown in Figure 1. *Azolla* is either protected with plastic sheets or housed in mud-roofed nursery beds in order to avoid frost damage. When temperatures get extremely cold, nurseries may also be covered with straw mats. In early spring, the *Azolla* is fertilized with farmyard manure, superphosphate, ammonium sulfate, and straw ash to accelerate its growth.[10]

A second overwintering technique is to pile *Azolla* into pits. Approximately 50 to 75 kg of *Azolla* is placed in a loosely piled stack which is covered with a removable cone-shaped A-frame. This is covered with 5 to 10 cm of straw ash and sprinkled with water to

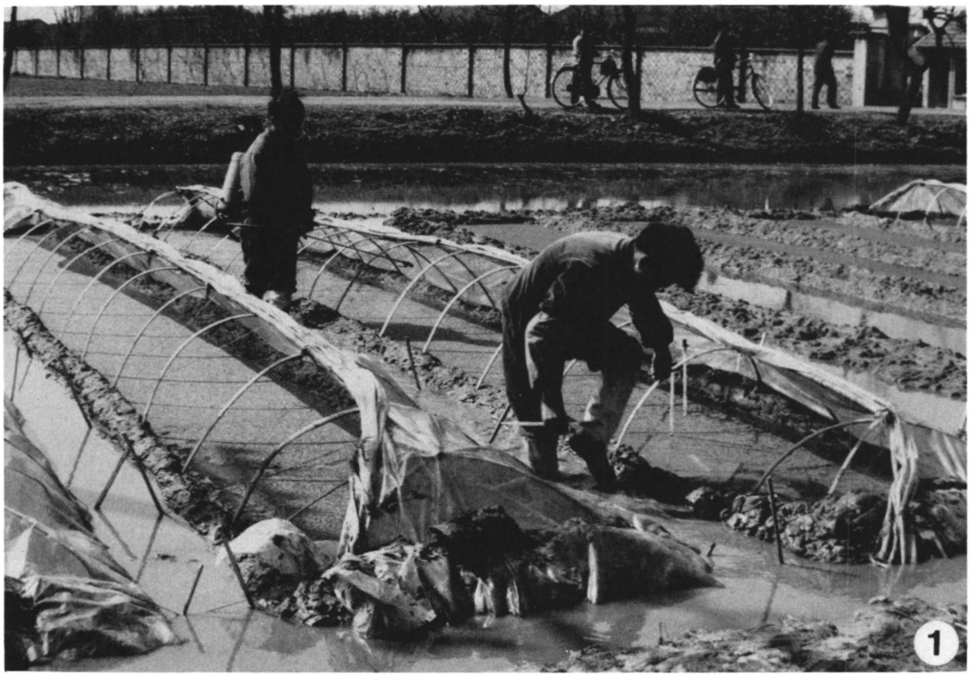

FIGURE 1. Arch-shaped overwintering nurseries constructed of bamboo strips and plastic sheets to protect *Azolla* against frost. The plastic sheets are pulled back during milder weather. Photograph courtesy of Thomas A. Lumpkin, Washington State University, Pullman.

prevent desiccation. Using this technique, 50 to 70% of the *Azolla* can be kept alive through winter.[3]

A third method is to use natural hot-spring ponds or fields irrigated with hot-spring water (13 to 15°C) for overwintering.[3] A variation of this method is to use warm, uncontaminated waste water from factories or steam plants.

Spring Nursery Propagation

The purpose of spring propagation is to multiply *Azolla* as early and as rapidly as possible. In China, spring propagation is from mid-March through mid-April. A doubling of *Azolla* biomass is obtained every 3 to 5 d, with the *Azolla* being continually subdivided. Once an *Azolla* covering two to three layers thick is obtained in the nursery field, a portion of the *Azolla* is transferred and used as the inoculum for adjoining fields.[10]

Oversummering of *Azolla*

Oversummering techniques are required in many regions to keep *Azolla* cool during the summer. Damage to *Azolla* from high water temperatures can be avoided by draining, shading, or flushing water.[1] Ideal sites for oversummering *Azolla* have good ventilation, partial shading, and cool running water. While ponds, canals, and streams are favored oversummering sites, field plots may also be used.[10] However, such plots must be irrigated with running water to keep temperatures low.[3] Low water levels during the day and irrigation of the fields at night help maintain low water temperatures. Partial shading for *Azolla* can be provided either by placing frames with loosely woven reed over the *Azolla* or by planting tall shade plants such as *Sesbania* or *Alternanthera* nearby.[10] Proper fertilization and regulation of nutrients increase the resistance of *Azolla* to hot water temperatures.[3] Also, it has been reported that spraying gibberellin on *Azolla* improves heat tolerance by reducing its respiration rate.[3]

The last and most important aspect of oversummering is the control of diseases, insects, and pests. It is crucial that *Azolla* to be oversummered be free of all pests. A detailed discussion of pests and pest control for *Azolla* is provided in the Subsection "Pest Control".

Autumn Nursery Propagation

Autumn nurseries are inoculated with *Azolla* taken from oversummering sites. At the beginning and end of the autumn propagation period, *Azolla* in the nurseries is supplied with phosphorus and small amounts of nitrogen fertilizer. In October or November, healthy *Azolla* plants are further propagated in preparation for overwintering.[10]

Cropping Systems

Basically, there are three different cropping systems. The specific system chosen varies each year depending upon the season, location, traditions, and other factors.[10] The cropping systems can be classified as: (1) *Azolla* grown as a monocrop (basal green manure), (2) *Azolla* grown as an intercrop and then incorporated as a topdressing, or (3) *Azolla* grown first as a monocrop and then as an intercrop.[10] The number of times *Azolla* is incorporated into the soil with these systems is varied. One study found that the best management practice was to grow *Azolla* as a monocrop, incorporate it into the soil once before transplanting, and then intercrop and topdress with *Azolla* once by death.[35]

In addition to choosing a cropping system, when using *Azolla* as a nitrogen fertilizer it is important to select appropriate rice varieties. The best varieties for intercropping with *Azolla* are compact types having straight, narrow leaves; hard stalks; big ears; a high number of grains; and medium tillering capacities.[3]

Azolla as a Monocrop: Basal Green Manure

Criteria for a good basal green manure include: (1) easily propagated, (2) fast growing, (3) high in nitrogen content, (4) easily and rapidly decomposed in soil, and (5) harmless to subsequent crops.[6] Because *Azolla* fulfills these criteria, it is no surprise that *Azolla* is frequently grown in rice fields as a monocrop basal manure prior to transplanting seedlings (Figure 2). If water is available in fields before rice planting, the fields are plowed, leveled, and irrigated. *Azolla* is then distributed in the fields, which are covered with 2 to 10 cm of water.[3,9] *Azolla* is grown in the fields for 10 to 30 d, after which it is incorporated into the soil one or two times as a basal green manure by plowing or harrowing.[1,10,29] Using *Azolla* as a green manure, it is possible to obtain a nitrogen accumulation of 2 kg/ha/d.[28]

Dual-Cropping (Topdressing)

In most areas, dual-cropping of rice and *Azolla* is the predominant method by which *Azolla* is applied as a nitrogen fertilizer (Figure 3). Dual-cropping usually follows a basal application of *Azolla*.

The procedure is quite simple. After basal application of *Azolla*, the rice seedlings are transplanted. A small amount of *Azolla* remains and is left to grow under the rice canopy. Full cover of *Azolla* is attained in 10 to 20 d.[3,12] *Azolla* is then incorporated into the soil by one of three techniques (see below). Again, any remaining *Azolla* is left to grow. Using this procedure, *Azolla* can be incorporated numerous times during dual-cropping. Incorporation usually occurs at 15, 25, and 55 d after planting.[3] The final *Azolla* covering dies naturally or is incorporated before the maximum tillering stage of rice. Hence, *Azolla* is used very effectively as a topdressing manure.[3,10,14]

Azolla dual-cropped with rice provides nutrients by decomposition after overgrowth or incorporation into the soil. As mentioned above, there are three basic techniques for incorporation of *Azolla* into the soil. The manual method involves turning *Azolla* into the soil by hand or with common farm tools such as weeders, shown in Figure 4.[1,9] Another method

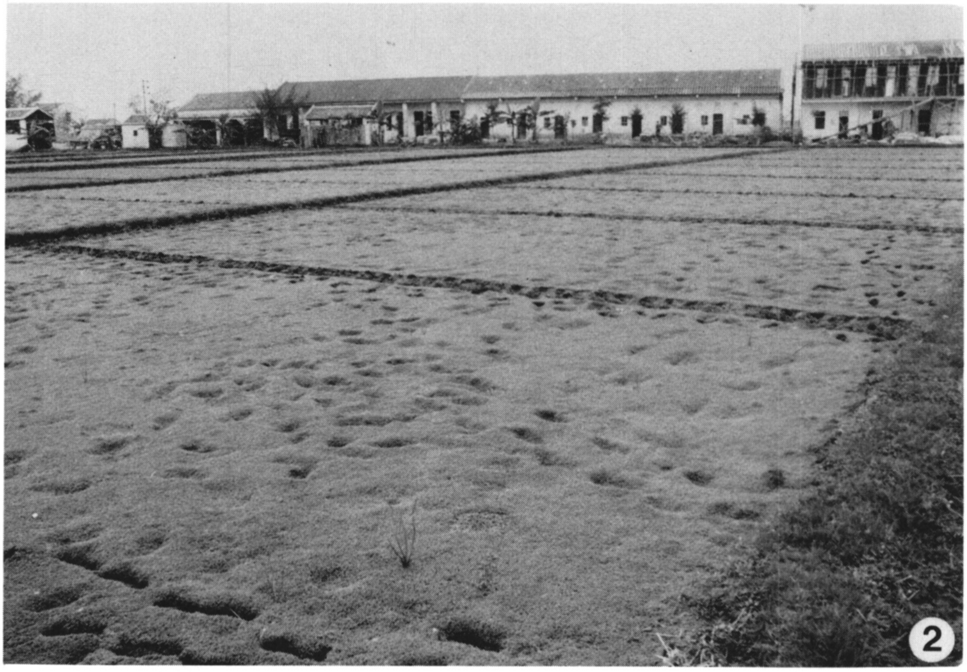

FIGURE 2. *Azolla* grown as a monocrop, basal green manure. Photograph courtesy of Thomas A. Lumpkin, Washington State University, Pullman.

is to chemically decompose *Azolla* using 2% urea, 2.5% ammonia liquor, or 44.1% Basagran.[3,14] A third method is simply to allow *Azolla* to decompose naturally. In general, *Azolla* fertilization is best when *Azolla* is incorporated into the soil just before planting.[3] Regardless of when *Azolla* is incorporated, the *Azolla* covering must die in order to get release of nitrogen from *Azolla* to the rice.[8]

An important consideration for dual-cropping is the spacing between rice rows. The traditional plant spacing is 18 × 15 cm. Nevertheless, by widening the distance between rows, *Azolla* can be grown continuously under a rice canopy.[36] Wider spaces are better for *Azolla* growth because more sunlight reaches the *Azolla*. In China, the spacing strategy used is called the double narrow-rows method.[3] In the double narrow-rows method, spacing between wide rows is 53 to 66 cm, between narrow rows is 13 cm, and between clusters is 6.5 cm.[3,31] *Azolla* is cultivated in the wide intervals. In Taiwan, the rice spacing is 25 × 20 cm. The spacing strategy that is used influences the method of *Azolla* incorporation and is also very important for *Azolla* growth.

Lastly, the density of the rice crop and the timing of *Azolla* and rice cultivation is important. The rice seedlings must be established before *Azolla* is allowed to form a full cover mat. Otherwise, the *Azolla* mat will have adverse effects on the rice by inhibiting its growth.[8] The partial shading of *Azolla* by developing rice does not appear to be a problem. In fact, shading may provide an optimal light intensity for the growth of *Azolla*.[28]

DECOMPOSITION AND INCORPORATION OF *AZOLLA*

Death and decomposition of *Azolla* to ammonia, which is then available as an inorganic ammonia fertilizer, is almost always the mode of nitrogen transfer to rice.[26] Organic nitrogen in *Azolla* is mineralized rapidly for the first 2 weeks, after which mineralization is more gradual.[1,8] Thus, nitrogen from *Azolla* is rapidly available to rice plants. In one study, peak release of ammonia occurred 7 d after incorporation, with release almost complete after 6

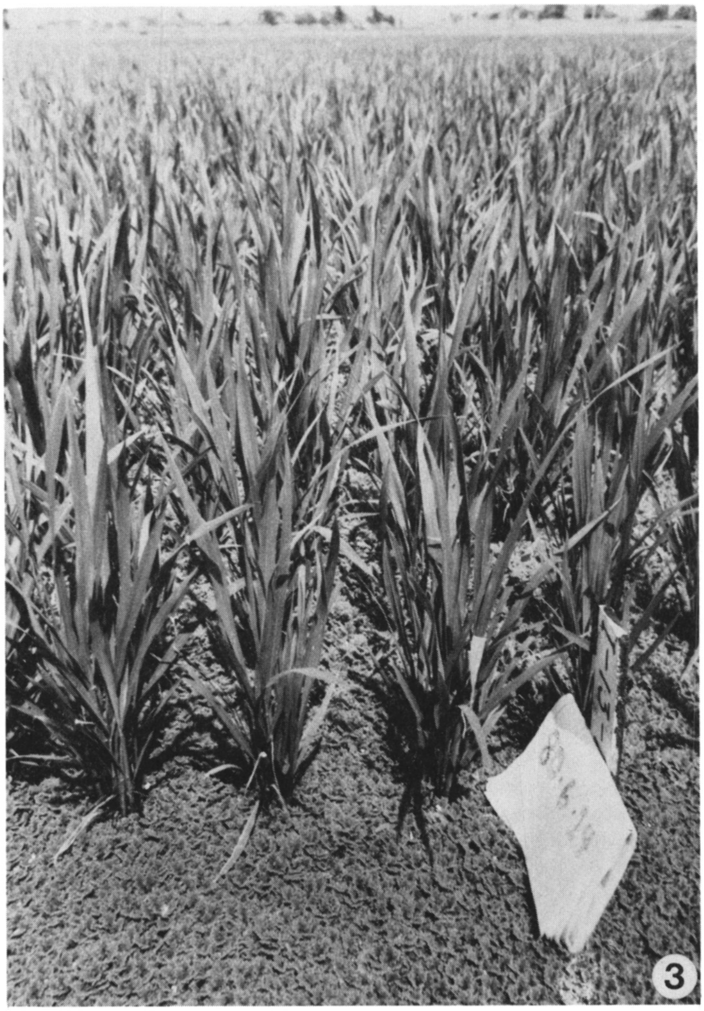

FIGURE 3. *Azolla* grown as an intercrop with rice. Photograph courtesy of Thomas A. Lumpkin, Washington State University, Pullman.

to 8 weeks.[12] Highest rice grain yields are obtained when the ammonia of *Azolla* is released at a maximum level within 10 to 15 d after rice transplantation. A key determinant in the rate of *Azolla* mineralization is the C to N ratio. A low C to N ratio of 10:1 favors very rapid decomposition and release of nitrogen within a few weeks after soil incorporation.[3,10]

Studies have been conducted to determine how long *Azolla* could remain incorporated in fallow flooded soil before significant losses of nitrogen occurred. It was found that nitrogen released from *Azolla* goes through a succession of ammonification, nitrification, and denitrification steps.[37] Decomposition of *Azolla* was followed using ^{15}N studies. Decomposition occurred in the following manner. Initially, $^{15}NH_4$ nitrogen was released, which was subsequently oxidized to $^{15}NO_3$ nitrogen in the surface aerobic layer. The $^{15}NO_3$ nitrogen diffused into the subsurface anaerobic layer, where it was denitrified to $^{15}N_2$.[37] Denitrification occurred after 30 d, which is the time required to develop a surface oxidized layer where nitrification can occur. $^{15}NH_4$ nitrogen generated in the bottom layer diffused up to the aerobic surface layer for nitrification. It was found that after 60 d, 30 to 32% of ^{15}N nitrogen from *Azolla* was released as mineral nitrogen, of which 93 to 96% was lost as N_2.[36,37] Thus, to avoid

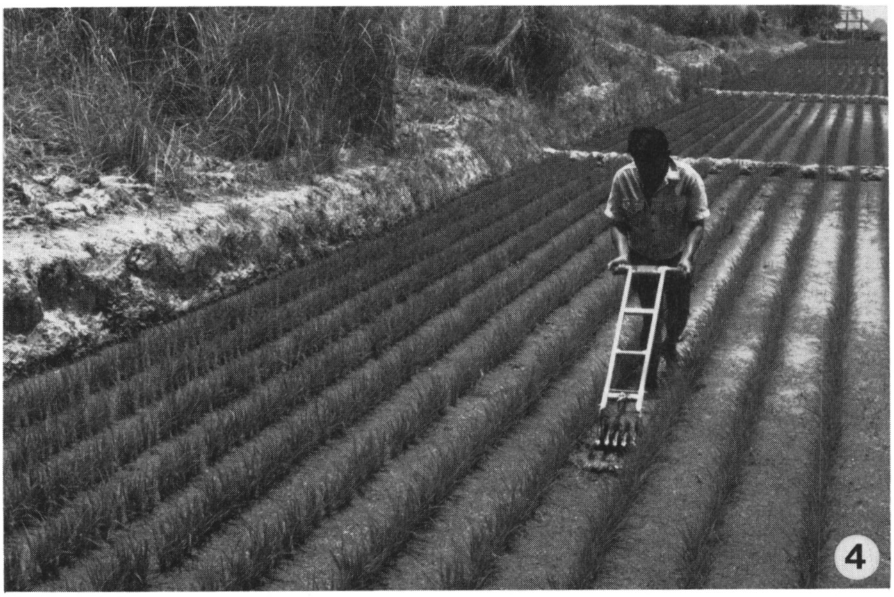

FIGURE 4. Incorporation of *Azolla* using a hand weeder by a Filipino farmer who first tried *Azolla* technology in the Philippines. Photograph courtesy of I. Watanabe, The International Rice Research Institute, Los Baños, Philippines.

severe denitrification loss of released *Azolla* nitrogen, it is necessary to plant rice seedlings within 3 weeks after *Azolla* incorporation into flooded soils.[37]

One study, in which *Azolla* nitrogen was released in the presence of rice plants, showed that 26% of ^{15}N from *Azolla* was released after 60 d. From this amount, 7% of the nitrogen was lost by denitrification and 74% stayed in the soil as undecomposed biomass. The remaining 19% was available for the rice plants.[36]

Fresh *Azolla* releases ammonia more rapidly than dried *Azolla*.[11,30] Dried *Azolla* is rapidly mineralized during the first 4 d, possibly due to small amounts of easily decomposable substances that are present in the dried *Azolla*.[30] Whereas dried *Azolla* incorporated at 50 kg N per hectare is equivalent to 15 kg N/urea, fresh *Azolla* at 200 g/m^2 is equivalent to 50 kg N/urea.[18] Thus, fresh *Azolla* is a better nitrogen fertilizer than dried *Azolla*.

During growth of *Azolla*, there is a very slow release of ammoniacal nitrogen directly into the water.[26] In one study, *Azolla* grown on the water surface supplied 10% of its original ^{15}N nitrogen to rice plants.[30] This ammoniacal nitrogen is presumably utilized by rice through its removal from solution by rice roots.[26] The contribution of this nitrogen to the total nitrogen input for rice plants is, however, very small.[38] Talley and Rains[26] postulated that leakage of nitrogen into the aquatic environment is triggered by environmental conditions leading to a depression of carbon fixation, but not nitrogen fixation. The amount of ammonia leakage is variable in different *Azolla* species. *Azolla pinnata* was found to release ammoniacal nitrogen more efficiently than either *A. filiculoides* or *A. mexicana*. Under laboratory conditions, the appearance of ammonia in culture medium was found to be closely related to the quality and quantity of light.[28] The specific factors that result in differential abilities of *Azolla* species to leak ammonia have not yet been determined. ^{15}N studies demonstrated that *Azolla* placed on the surface of either water or soil has the same degree of nitrogen availability.[30]

Last, there has been one report that nitrogen supplied from sloughed-off root tissues may contribute to the increase in rice yield.[30]

ADDITIONAL APPLICATIONS OF *AZOLLA* AS A NITROGEN SOURCE

Preparation of Mud Manure

Azolla has recently been used in Hunan and Kiangsu provinces to produce fertile soil. Initially, a fallow field (between the first and second rice crop) is plowed. *Azolla* is inoculated into the field and allowed to grow to confluency, after which the *Azolla* is incorporated into the soil. *Azolla* is then grown again, turned into the soil again, etc. This process is repeated four to six times. After the final *Azolla* incorporation, the upper 10 cm of soil is removed and used as fertile soil in other locations. The remaining top 6 cm of soil is left as fertile soil for the subsequent crop. Using this procedure, 1 ha can produce enough fertile soil to supply basal manure for 10 ha of late rice or wheat.[3]

Azolla in Field Rotation Systems

In North Vietnam, *Azolla* is used in rice rotations in a manner similar to that of legumes in upland crop rotations.[2,12] In these areas, *Azolla* is a better rotation crop for rice than soybean due to greater tolerance to cold temperatures. Basically, *Azolla* is used in field rotations after two crops of rice. The spring rice is grown from February through June, and the summer rice is grown from July through October/November. Between mid-October and December, an early *Azolla* crop is grown (the ''green'' *Azolla* species). From mid-December through February a late *Azolla* crop is grown. The ''purple'' *Azolla* species is used as the late crop because it is better adapted to low sunlight and cooler temperatures.[12,31] *Azolla* has also been used as a fallow season (grown November to April) green manure crop which is incorporated into the soil during spring field preparation.[26]

Azolla as a Green Manure for Upland Soils

In Vietnam, *Azolla* is also used as a green manure for upland soils.[12,39] When used for this purpose, *Azolla* not only supplies a nitrogen source, but also helps retain moisture for the crop. In general, cultivation of *Azolla* improves both the physical conditions of the soil and soil fertility.[3] When used as a basal manure, *Azolla* can benefit growth of wheat and rape.[3] In one study it was shown that if 20 to 30 tons of *Azolla* was applied to an upland nonirrigated crop field, planted seeds germinated 2 to 3 d earlier than if no *Azolla* was applied.[12]

Azolla has also been successfully used as a green manure for corn in Mexico.[17] *Azolla* incorporated into the soil as a green manure was found to be a feasible alternative to chemical nitrogen fertilizers, increasing the growth of maize.[17] *Azolla* is also used as a green manure for water bamboo, arrowhead, and taro.[10,39]

Topdressing

In some regions, *Azolla* is grown for use as a topdressing in low-lying land where water stagnates. Once the rice is at a relatively late stage of growth, either fresh or slightly decomposed *Azolla* that was grown in low-lying land can be added as a topdressing.[9]

ADDITIONAL USES OF *AZOLLA*

AZOLLA AS ANIMAL FODDER

Azolla is rich in proteins, fats, and amino acids, and has been used as a fodder for pigs,[2,3,12,31] cattle,[2,31] poultry,[2,8,12] sheep,[40] fish,[31,39] ducks,[31,39] and buffalo.[12] It has even been suggested as food for humans.[9] The high protein content in *Azolla* makes it attractive as a fodder.[12,28] However, the high fiber content of *Azolla* restricts efficient use as a protein source for monogastric animals.[31,41] The digestibility of *Azolla* is affected by its age. *Azolla* is 77% digestible during exponential growth and 49% digestible after it has aged.[40] When used as a fodder, *Azolla* can be served fresh, cooked, fermented, or dried.[3] In dried form, *Azolla* has been substituted for 15 to 20% of the starchy feed required for pigs.[12] In another report, the addition of *Azolla* powder to poultry feed significantly improved the carotene status of chickens and increased egg production.[12] Livestock gained weight faster when fed an *Azolla*-rice straw mixture than when fed rice straw alone.[12]

Azolla is currently used as fodder in North Vietnam,[8,12,28] Asia, Africa,[9] and China.[3] Often, *Azolla* is grown in the winter season when fodder is scarce. Grown on 1 ha of land, 2.09 tons of *Azolla* produce the equivalent to 27.6 kg of protein.[3] Hence, the potential use of *Azolla* as a food source appears to be very promising.

AZOLLA AS A WEED SUPPRESSOR

Azolla is also valuable as a weed suppressor when grown in dual culture with rice.[3,8,13,14,28,31] Rains and Talley[28] reported that early growth of *A. filiculoides* smothered the growth of *Cyperus* and *Polygonum* weed species from rice paddies. However, the *Azolla* cover did not reduce growth of *Echinochloa*, a taller weed. Up to 80% weed reduction due to the presence of *Azolla* has been reported.[13] *A. filiculoides* has also been used by Hawaiian taro growers to suppress growth of weeds.[42]

IMPROVEMENT OF WATER QUALITY USING *AZOLLA*

High concentrations of phosphorus and nitrates significantly contribute to the deterioration of water quality. Thus, it has been suggested that biological systems may be useful for improving water quality by removing nutrients from the water. *Azolla* might be useful for this purpose because it can take up nitrates and is readily harvested by skimming it off the water surface.[28] There is one report in support of this idea, in which water drawn from a canal with a population of *Azolla* was easier to purify than a control. It was suggested that this indicated that impurities in the canal water had been reduced by the *Azolla*.[43]

OPTIMIZING GROWTH AND NITROGEN FIXATION OF *AZOLLA*

There are a number of environmental factors that influence the growth and nitrogen-fixing capabilities of *Azolla*. Collectively, factors such as light intensity, humidity, nutrient availability, pH, water depth, winds, and temperature determine the nitrogen-fixing capabilities of *Azolla*. These factors have been discussed in detail in the preceding chapter.

NUTRIENT REQUIREMENTS OF *AZOLLA*

Phosphorus

Although *Azolla* is a good source of nitrogen fertilizer for rice, in some regions the phosphorus content of soil or paddy water is too low to meet the phosphorus requirement of *Azolla*. Thus, *Azolla* often requires a phosphorus fertilizer.[21,29]

In the laboratory, 0.06 ppm phosphorus is required for normal growth of *A. pinnata* in continuous culture.[44] However, in the field, 0.3 to 1.0 ppm phosphorus as P_2O_5 is the required phosphorus concentration for fulfilling the growth requirements of *Azolla*.[21,45] The exact phosphorus requirement depends upon soil characteristics, including the phosphate absorbing capacity. It was reported that *A. pinnata* could grow without added phosphorus in soils with Olsen phosphorus values higher than 30 mg/kg and phosphorus sorption capacities lower than 1500 mg P_2O_5 per 100 g.[46]

Phosphorus Deficiency in Azolla

Symptoms of phosphorus deficiency in *Azolla* include reduced growth, decreased size, fragile fronds, browning and elongation of roots, and reddening/browning of dorsal and ventral leaf lobes.[9,31,44] In *A. mexicana*, browning starts at the newer leaves, whereas in other species browning starts at the older leaves.[31,45] The significance of this is unknown. In regard to the endosymbiont, phosphorus deficiency causes a reduction in the number of heterocysts. *Anabaena* cells become pale green due to reduced chlorophyll content, vegetative cells and heterocysts become extremely long,[45] and there is an overall decrease in nitrogenase activity.[44] It has been reported that phosphorus deficiency can cause a 50% decrease in the nitrogen content of *Azolla*.[12]

It should also be noted that there is a significant difference in the ability of *Azolla* species to assimilate and utilize phosphorus.[45] Rates of phosphorus uptake and utilization are lowest in *A. caroliniana* and *A. filiculoides*. Under phosphorus-limiting conditions, *A. pinnata* grows better than the New World species.[44] Rains and Talley speculated that species with better root development exhibit greater phosphorus uptake,[45] but experimental evidence in support of this hypothesis has not yet been obtained.

Phosphorus Fertilization of Azolla

The best phosphorus fertilizer is soluble superphosphate (P_2O_5).[2,3,12] Citrate soluble phosphate, calcium magnesium phosphate,[3] and fused magnesium phosphate[2] are alternate fertilizer choices. Insoluble rock and Ratna phosphates cannot be used as fertilizers[2,9] unless they have been finely ground.[12] In some instances, manure or urine can be used.[2] It has been estimated that 1 kg of P_2O_5 fertilizer results in 2 kg additional nitrogen in *Azolla* biomass.[21] Apparently, *Azolla* obtains phosphorus from the water.[28]

Usually, phosphorus fertilizer is applied in split applications rather than a single basal application. Split applications greatly reduce the total amount of fertilizer required.[21,31] Split applications may be better due to a lesser stimulation of growth of competing green or blue-green algae. At higher phosphorus concentrations (used for a single application), competing organisms grow rapidly and may inhibit the growth of *Azolla*.[28] Recommended amounts of phosphorus fertilizers for basal and split applications in different geographical areas are listed in Table 5. It should be noted that *Azolla* grown during the fallow season requires more phosphorus than that grown in dual-culture with rice in the summer.[29] It has been suggested that *Azolla* might require more phosphorus during the fallow season due to cold temperatures, which may restrict the ability of *Azolla* to acquire phosphorus.[47]

Iron

The critical concentration of iron for growth of *Azolla* is 20 μg of iron per liter.[28] Although the iron concentration in flooded rice fields is usually sufficient for growth of *Azolla*, in some instances an iron fertilizer may be required. Iron-deficient *Azolla* contains a reduced chlorophyll content, which results in *Azolla* turning yellowish.[11] To eliminate iron deficiencies it is suggested that 50 g of iron as Fe-EDTA be applied per hectare every 10 d.[26] Alternatively, if the soil is flooded before inoculation of *Azolla*, iron from the reduced soil layer becomes available.[31]

Potassium

In general, potassium fertilizer for growth of *Azolla* is not required. However, application of fertilizer may be required for light texture soils when the clay content is less than 10%.[12,48] Potassium-deficient *Azolla* plants are yellowish/brown and have a tenfold lower potassium content than healthy plants.[31] When required, potassium sulfate, potassium magnesium, ash, muriate potash,[8] or farmyard manure can serve as the fertilizer. An application of 5 kg K per hectare every 5 to 7 d is suggested.[12] Alternately, 10 kg K_2O or 100 kg ash per hectare every 10 d has been suggested.[2] In Taiwan, two applications of KCl are used; 60% of the KCl fertilizer is applied as a basal application, with the remaining 40% applied 30 d after rice seedlings are planted.[14]

Molybdenum

Azolla requires 0.7 ppm molybdenum for good growth and nitrogen fixation. Most soils contain this amount of molybdenum. Therefore, molybdenum fertilizers are rarely required.

TABLE 5
Phosphorus Fertilization (Recommended Amounts)

Location	Amount of fertilizer (kg/ha)	Type of fertilizer	Timing and no. of applications	Ref.
North America	30	P_2O_5	1 day after inoculation	26
	5	P_2O_5	Split application at 1, 10, 20 days after inoculation, totaling 15 kg/ha	26
North America				
A. filiculoides	60	P_2O_5	Fallow season, 1 application	28
	15	P_2O_5	Fallow season, split application, 2 times, totaling 30 kg/ha	28
A. mexicana	5	P_2O_5	Fallow season, split application at 1,10, 21 days after inoculation, totaling 15 kg/ha	28
Vietnam	5	P_2O_5	Every 5 days	1
Vietnam	5—10	P_2O_5	Every 5 days	2
	125—250	Urine	Every 5 days	2
	1000	Manure	Every 10 days	2
Vietnam	5	P_2O_5	Every 5—7 days	12
Philippines	7.5	P_2O_5	Every 2—4 days (recommended)	21
	1	P_2O_5	Every 2—4 days (minimum requirement)	21
India	4—8	P_2O_5	Spread or mixed with *Azolla* inoculum	9
China	187.5	P_2O_5	Basal manure, 1 application	3
	22.5	2% P_2O_5 solution	Sprayed on *Azolla* leaves every 5—6 days, totaling 22.5 kg	3
Taiwan	15	P_2O_5	Basal manure, 3 applications, totaling 45 kg/ha	14
	80	P_2O_5	Dual-cropping, 1 application	14

PEST CONTROL

Insects

During the hot, humid, and rainy seasons, rapid insect and fungal growth is an obstacle for growing *Azolla*.[1,2,9] Problems with insects are most severe during summer months when temperatures are higher than 30°C. Problems arise because the generation time of insects decreases as temperatures increase.[29,31] Insects can damage an entire *Azolla* crop in only a few days.[9] The major insect pests and recommended insecticides for different geographical areas are listed in Table 6. The reader is referred to Liu[3] and Watanabe[31] for a detailed account of the infection process for a number of *Azolla* pests. Recommended concentrations of insecticides used for the control of *Azolla* pests are also presented by Liu.[3]

During summer months and in the tropics, pests are a major constraint for growing *Azolla*. Thus, good pest control is a key to oversummering *Azolla* in hot climates. While a number of insecticides are currently used to control pests, unless more effective methods of pest control are developed or chemical prices decrease, *Azolla* will never be economically feasible in areas of frequent insect outbreaks.[29] One study reported that if greater than 200 g.a.a. carbofuran per hectare is required to control insects and pests, then the economic benefits gained from the reduction of fertilizer costs are lost.[29]

Fungi

In hot climates, damage of *Azolla* by fungal attack is also a serious problem. Fungal attack often follows damage by insects or desiccation.[29,31] In Africa, small *Lebistes* fish

TABLE 6
Major Insect Pests and Recommended Insecticides

Location	Insects/Pests	Effect on Azolla	Insecticide/treatment	Ref.
Vietnam	*Pyralis* larvae	Lays eggs between dorsal and ventral lobes of *Azolla*; larvae form burrows and feed on *Azolla* leaf buds	DDT or BHC	2, 12
	Nymphula larvae	Rolls and eats buds and lobes of *Azolla*	Ethyl or methyl parathion solution, BHC, or DDT suspension	2, 12
	Chironomas larvae (most destructive)	Attacks growing buds and lobes of *Azolla*	Ethyl parathion solution or DDT suspension	2, 12
China	*Pyralis* larvae (most destructive)	Lays eggs between dorsal and ventral lobes of *Azolla*; larvae form burrows and feed on *Azolla* leaf buds	Padan, malathion, sumithion, carbofuran, dichlorvos	3, 31
	Nymphula larvae (brown and Black species)	Lays eggs on back side of ventral lobe of *Azolla*; the larvae attack and eat *Azolla*	Padan, malathion, sumithion, carbofuran, dichlorvos	3, 31
	Chironomas larvae (thread worms)	Feeds on growing buds, lobes, and roots of *Azolla*	Fenithion, carbofuran, actinomycete culture	3, 31
	Lepidopterous larvae		Baccilicide	3, 31
	Bagous sp. (snout beetle)	Feeds on leaf or leaf bud of *Azolla*; bores into the stem-leaf, segregating the *Azolla* body	Dichlorvos, dipterex	3, 31
	Radix swinhoer (Azolla snail)	Feeds on leaves and roots of *Azolla*; damages *Azolla* body which becomes segregated and rootless	Tea oil seed cake, dichlorvos, dipterex	3, 31
India	*Lepidopterous* larvae; *Dipterous* larvae	Rolls leaves of *Azolla* and feeds on them	Savidolor, Furadon, BHC, sevidol, carbofuran	8,9
Taiwan			Furadon, orthene	13

damage *Azolla*, which facilitates fungal attack.[18] The major fungal pests are *Sclerotium* and *Rhizoctonia*.[30,31] The fungal pathogen *Rhizoctonia* species is found with *Azolla* everywhere in the world under hot, humid conditions.[49,50] Benomyl, PCNB, Ceresan, and Nirit can be used to control *Rhizoctonia*. Spraying infected plants with an ethylmercuric chloride solution or with a dinitrophenyl thiocyanate solution is also an effective treatment.[10] However, it should be noted that if large amounts of fungicides are needed, the cost of *Azolla* production becomes unprofitable and poses a serious environmental threat to fish.[51]

CONSTRAINTS OF USING *AZOLLA*

While in many areas *Azolla* is a very attractive alternative to chemical nitrogen fertilizers, there are some constraints for its use that should be mentioned.

In Vietnam, the main constraint for expanding use of *Azolla* is temperature. Temperatures in the south are too high and those in the north are too low.[1,12] The very hot summer temperatures make the use of *Azolla* uneconomical due to poor growth. This is partially due to severe insect damage. Consequently, *Sesbania* is used for the summer rice. An additional problem in South Vietnam is the lack of irrigation water which makes it impossible to use *Azolla*. However, there is year-round water in northern Vietnam so that two crops of rice and one crop of *Azolla* can be grown.[2] Floods and storms, coupled with heavy rains in the summer and autumn, may also restrict the use of *Azolla* in some parts of Vietnam.

In Taiwan, in addition to temperature constraints, there are other factors which limit the use of *Azolla*. Major constraints are that (1) rotation-irrigation systems make it difficult to maintain water in paddies during dual-culture of rice and *Azolla* and (2) herbicides have been used frequently in rice fields, which severely damaged *Azolla*.[13] Also, farmers have used chemical nitrogen fertilizers for a long time (and are therefore somewhat resistant to change) and labor costs are expensive. Nevertheless, energy shortages in Taiwan make it difficult to produce large amounts of chemical fertilizer, and this makes the use of *Azolla* more attractive.[13]

In Asian tropical rice fields, a general constraint for the use of *Azolla* is that only 20% of the fields have adequate water control. This is a major problem, since *Azolla* is very sensitive to drought and will die after a 1- or 2-d drought.[1]

In North America, high labor costs reduce the likelihood that *Azolla* will be used in rice culture in the immediate future.[28] In Indonesia, the use of *Azolla* is limited because nitrogen fertilizers are available at relatively inexpensive subsidized prices.[6]

Lastly, there are a few reported problems associated with the use of *Azolla* that may restrict its use in specific locations. Decomposition of *Azolla* in areas other than rice fields during the summer months has been identified as a potential problem. This decomposition may provide nutrients to noxious weeds.[8] Also, it has been reported that heavy *Azolla* blooms can create an unhealthy environment for fish.[8]

ECONOMIC CONSIDERATIONS

Only a few reports on the economic feasibility of using *Azolla* exist.[1,29] The economic potential of *Azolla* is greatest in countries where the cost of labor is low ($12/d or less).[30] Thus, *Azolla* has great economic potential in India, Bangladesh, and Indonesia, with less potential in Taiwan, some Provinces of China, and Korea, where rural wage rates are high. Estimates of cost savings through use of *Azolla*, rather than chemical nitrogen fertilizers, range from $10 to 37 per hectare, 1982 value.[1] While the labor required for applying *Azolla* is 2.8 h/ha, it is suggested that the reduction in costs of chemical fertilizer application produce reductions in labor which almost cancel the higher labor requirements of *Azolla* application.[29] It should also be noted that the exact labor requirements vary greatly, depending upon specific management practices and specific growth conditions. In addition, the fact that labor costs for *Azolla* could open new employment opportunities in the "rice section" must also be considered.[29] Reduced weeding costs through the use of *Azolla* have also been considered.

CONCLUSIONS AND FUTURE RESEARCH

Based on the preceding discussion, a number of areas requiring further research can be identified. In order to extend the use of *Azolla* in agriculture, the search for and development of strains that are well suited for specific geographical regions is required. Development of strains which are better nitrogen fixers, tolerant of temperature extremes, and resistant to diseases and pests will be particularly important.[9,17] Additionally, *Azolla* strains that can either tolerate phosphorus-deficient conditions or scavenge phosphorus from the water and soil efficiently will be extremely valuable.[12] Also, intraspecific crossbreeding of *Azolla* may be useful in extending the range of conditions under which *Azolla* can be cultivated.[10]

A better understanding of nutrient requirements and timing of fertilizer application is required to obtain maximal production of *Azolla*.[28] More efficient fertilization techniques for different soil types in different locations need to be established. Also, research on the decomposition and accumulation of organic matter in *Azolla* is required.

Production, management practices, and transport of *Azolla* continue to be areas requiring further study. Research should include improving cultural techniques that would allow *Azolla* to grow under less favorable conditions. Also, more efficient methods of incorporating *Azolla* into the soil when grown as an intercrop with rice need to be developed.[10] The weed control aspects of *Azolla* growth should receive further attention, and may turn out to be an important management tool when using *Azolla* as an intercrop.[28]

An area in which very little research has been conducted is that related to the sexual reproduction of *Azolla*. A large portion of the labor and land costs involved with *Azolla* cultivation could be reduced if control and management of sporulation in *Azolla* were better understood. Effective methods of producing and collecting large amounts of spores, as well as quick and simple methods of breeding plantlets from spores, are needed. Storage of spores, rather than *Azolla* plantlets, would eliminate many of the problems associated with overwintering and oversummering *Azolla*.

Lastly, it has been suggested that utilization of *Azolla* as a fodder should be extended.[12]

REFERENCES

1. **Watanabe, I.**, Use of symbiotic and free-living blue-green algae in rice culture, *Outlook Agric.*, 13(4), 166, 1984.
2. **Tuan, D. T. and Thuyet, T. Q.**, Use of *Azolla* in rice production in Vietnam, in *Nitrogen and Rice*, International Rice Research Institute, Los Baños, Philippines, 1979, 395.
3. **Liu, C. C.**, Use of *Azolla* in rice production in China, in *Nitrogen and Rice*, International Rice Research Institute, Los Baños, Philippines, 1979, 375.
4. **Moore, A. W.**, *Azolla*: biology and agronomic significance, *Bot. Rev.*, 35, 17, 1969.
5. **Whitton, B. A. and Catling, H. D.**, Algal ecology of deepwater rice-fields in Thailand, *Arch. Hydrobiol.*, 105(3), 289, 1986.
6. **Brotonegoro, S., Sudjadi, M., Partohardjono, S., Sukiman, H., Prihatini, T., and Hendriks, V.**, Some experiments on the use of *Azolla* for rice production in Indonesia, in *Biological Nitrogen Fixation Technology for Tropical Agriculture*, Graham, P. H. and Harris, S. C., Eds., Centro International de Agricultura Tropical, Cali, Colombia, 1982, 567.
7. **Becking, J. H.**, Nitrogen fixation in some natural ecosystems in Indonesia, in *Symbiotic Nitrogen Fixation in Plants*, Nutman, P. S., Ed., Cambridge University Press, Cambridge, 1975, 539.
8. **Singh, P. K.**, Multiplication and utilization of fern "*Azolla*" containing nitrogen-fixing algal symbiont as green manure in rice cultivation, *Riso*, 26, 125, 1977.
9. **Singh, P. K.**, Use of *Azolla* rice production in India, in *Nitrogen and Rice*, International Rice Research Institute, Los Baños, Philippines, 1979, 407.
10. **Lumpkin, T. A.**, Chinese technology for the cultivation of *Azolla*, in *Biological Nitrogen Fixation Technology for Tropical Agriculture*, Graham, P. H. and Harris, S. C., Eds., Centro International de Agricultura Tropical, Cali, Colombia, 1982, 537.
11. **Watanabe, I., Espiñas, C. R., Berja, N. S., and Alimagno, B. V.**, Utilization of the *Azolla-Anabaena* Complex as a Nitrogen Fertilizer for Rice, Int. Rice Res. Paper Ser. No. 11, 1977.
12. **Anon.**, The Research Center of Azolla Hanoi, Vietnam, Propagation and agricultural use of *Azolla* in Vietnam, *Int. Rice Comm. Newsl.*, 34(1), 79, 1985.
13. **Lee, C. C. and Lin, C. J.**, The possibility of using *Azolla* as a source of nitrogen for rice in Taiwan, *Soils and Fertilizers in Taiwan*, p. 35, 1981.
14. **Lin, C. J. and Lee, C. C.**, Using *Azolla* as a source of nitrogen for rice in Taiwan, in *Nitrogen and Rice*, International Rice Research Institute, Los Baños, Philippines, 1979, 83.
15. **Fiore, M. D. F. and Ruschel, A. P.**, The *Azolla Anabaena-Azollae* association. I. Biology and significance in agriculture, *Cien. Cult.*, 34(6), 792, 1982.
16. **Espinosa Abarca, S. A., Mayorga, S. P., and Ortega, M. M.**, A study on the growth of *Azolla filiculoides* in culture media and in rice field soil of Morelos state Mexico, *Rev. Latino-am. Microbiol.*, 27, 61, 1985.
17. **Ferrera-Cerrato, R. and Romero, A. M.**, Propagation of an *Azolla* sp. and its potential as a green manure for corn in Mexico, in *Biological Nitrogen Fixation Technology for Tropical Agriculture*, Graham, P. H. and Harris, S. C., Eds., Centro International de Agricultura Tropical, Cali, Colombia, 1982, 561.
18. **Reynaud, P. A.**, The use of *Azolla* in West Africa, in *Biological Nitrogen Fixation Technology for Tropical Agriculture*, Graham, P. H. and Harris, S. C., Eds., Centro International de Agricultura Tropical, Cali, Colombia, 1982, 565.
19. **App, A. A., Watanabe, I., Alexander, M., Ventura, W., Daez, C., Santiago, T., and De Datta, S. K.**, Non-symbiotic nitrogen fixation associated with the rice *Oryza-Sativa* plant in flooded soils, *Soil Sci.*, 130(5), 283, 1980.

20. **Ventura, W. and Watanabe, I.,** N-15 dilution technique of assessing the contribution of nitrogen fixation to rice plant, *Soil Sci. Plant Nutr.*, 29, 123, 1983.
21. **Watanabe, I., Berja, N. S., and Del Rosario, D. C.,** Growth of *Azolla* in paddy field as affected by phosphorus fertilizer, *Soil Sci. Plant Nutr.*, 26(2), 301, 1980.
22. **Becking, J. H.,** Symbiosen: Stickstoff-Bidung, *Fortschr. Bot.*, 34, 459, 1972.
23. **Becking, J. H.,** Contribution of plant-algal associations, in *Proc. 1st Int. Symp. Nitrogen Fixation,* Vol. 2, Newton, W. E. and Nyman, C. J., Eds., Washington State Press, Pullman, 1976, 556.
24. **Becking, J. H.,** Environmental requirements of *Azolla* for use in tropical rice production, in *Nitrogen and Rice,* International Rice Research Institute, Los Baños, Philippines, 1979, 345.
25. **Talley, S. N. and Rains, D. W.,** *Azolla filiculoides* Lam. as a fallow season green manure for rice in a temperate climate, *Agron. J.*, 72, 11, 1980.
26. **Talley, S. N. and Rains, D. W.,** *Azolla* as a nitrogen source for temperate rice, in *Nitrogen Fixation,* Vol. 2, Newton, W. E. and Orme-Johnson, W. H., Eds., University Park Press, Baltimore, 1980, 311.
27. **Watanabe, I.,** Biological nitrogen fixation by *Azolla-Anabaena* symbiosis and its utilization, *J. Sci. Soil Manure,* 52(5), 455, 1981.
28. **Rains, D. W. and Talley, S. N.,** Uses of *Azolla* in North America, in *Nitrogen and Rice,* International Rice Research Institute, Los Baños, Philippines, 1979, 419.
29. **Kikuchi, M., Watanabe, I., and Haws, L. D.,** Economic evaluation of *Azolla* use in rice production, in *Organic Matter and Rice,* International Rice Research Institute, Los Baños, Philippines, 1985, 569.
30. **Ito, O. and Watanabe, I.,** Availability to rice plants of nitrogen fixed by *Azolla, Soil Sci. Plant Nutr. (Tokyo),* 31(1), 91, 1985.
31. **Watanabe, I.,** *Azolla-Anabaena* symbiosis — its physiology and use in tropical agriculture, in *Microbiology of Tropical Soils and Plant Productivity,* Dommergues, Y. R. and Diem, H. G., Eds., Martinus Nijhoff, The Hague, 1982, 169.
32. **Talukdar, H. and Barthakur, H. P.,** Biomass production of *Azolla* and its effect on rice production, *J. Res. Assam Agric. Univ.*, 3(2), 123, 1982.
33. **Mian, M. H. and Stewart, W. D. P.,** A ^{15}N tracer study to compare nitrogen supply by *Azolla* and ammonium sulphate to IR8 rice plants grown under flooded conditions, *Plant Soil,* 83, 371, 1985.
34. **Ashton, P. J.,** The effect of some environmental factors on the growth of *Azolla filiculoides* Lam., in *The Orange River Progress Report,* van Zinderen-Bakker, E. M., Ed., University of the Orange Free State, Bloemfontein, South Africa, 1974, 123.
35. **Lumpkin, T. A., Zhuo-zing, L., Shou-xian, Z., and Mei-fei, M.,** The effect of species of *Azolla* under three management practices on the yield of paddy rice, in *Biological Nitrogen Fixation Technology for Tropical Agriculture,* Graham, P. H. and Harris, S. C., Eds., Centro International de Agricultura Tropical, Cali, Colombia, 1982, 549.
36. **Mian, M. H. and Stewart, W. D. P.,** Fate of nitrogen applied as *Azolla* and blue-green algae (Cyanobacteria) in water logged rice soils — a ^{15}N tracer study, *Plant Soil,* 83, 363, 1985.
37. **Mian, M. H.,** Detection of denitrification, by a ^{15}N tracer technique, of nitrogen released from *Azolla* and blue-green algae in a flooded soil, *Aust. J. Soil Res.*, 23, 245, 1985.
38. **Singh, P. K., Panigrahi, B. C., and Satapathy, K. B.,** Comparative efficiency of *Azolla,* blue-green algae and other organic manures in relation to N and P availability in a flooded rice soil, *Plant Soil,* 62, 35, 1981.
39. **Lumpkin, T. A. and Plucknett, D. L.,** *Azolla*: botany, physiology, and use as green manure, *Econ. Bot.*, 34(2), 111, 1980.
40. **DeWaha Baillonville, T., Godard, P., and Van Hove, C.,** Chemical composition of *Azolla* populations as affected by aging, *Arch. Int. Physiol. Biochim.*, 92, 30, 1984.
41. **Buckingham, K., Ela, S., Morris, J., and Goldman, C.,** Protein quality evaluation and proximate analysis of *Azolla filiculoides, Fed. Proc. Am. Soc. Exp. Biol.*, 36, 4802, 1977.
42. **Fosberg, F. R.,** Use of Hawaiian ferns, *Am. Fern J.*, 32, 15, 1942.
43. **Cohn, J. and Renlund, R. N.,** Notes on *Azolla caroliniana, Am. Fern J.*, 43, 7, 1953.
44. **Subudhi, B. P. R. and Watanabe, I.,** Differential phosphorus requirements of *Azolla* species and strains in phosphorus-limited continuous culture, *Soil Sci. Plant Nutr. (Tokyo),* 27(2), 237, 1981.
45. **Rains, D. W. and Talley, S. N.,** Use of *Azolla* in North America, in *Nitrogen and Rice,* International Rice Research Institute, Los Baños, Philippines, 1979, 419.
46. **Watanabe, I. and Ramirez, C. M.,** Relationship between soil phosphorus availability and *Azolla* growth, *Soil Sci. Plant Nutr. (Tokyo),* 30(4), 595, 1984.
47. **Sutton, C. D.,** Effect of low soil temperature on phosphate nutrition of plants, a review, *J. Sci. Food Agric.*, 20, 1, 1969.
48. **Talley, S. N., Talley, B. J., and Rains, D. W.,** Nitrogen fixation by *Azolla* in rice field, in *Genetic Engineering for Nitrogen Fixation,* Hollaender, A., Ed., Plenum Press, New York, 1977, 259.
49. **Shahjahan, A. K. M., Miah, S. A., Nahar, M. A., and Majid, M. A.,** Fungi attack *Azolla* in Bangladesh, *Int. Rice Res. Inst. Newsl.*, 5(1), 17, 1980.

50. **Venkataraman, S. and Kannaiyan, S.,** Control of black rot disease of *Azolla, Int. Rice Res. Newsl.,* 9(2), 13, 1984.
51. **Lumpkin, T. A. and Bartholomew, D. P.,** Crop physiology and metabolism: predictive models for the growth response of eight *Azolla* accessions to climatic variables, *Crop Sci.,* 26(1), 107, 1986.

Chapter 6

THE CYCAD-CYANOBACTERIAL SYMBIOSIS

Peter Lindblad and Birgitta Bergman

THE CYCAD PLANT

The cycad is the only example of a symbiosis between a gymnosperm and an organism capable of nitrogen fixation. Although palm-like in appearance, cycads are gymnosperms that differ from flowering plants, the angiosperms, by producing ovules borne naked on a scale of the cone. As a group, cycads had their largest geographical distribution 100 million years ago. Today there are approximately 150 cycad species belonging to the families Cycadaceae (*Cycas*), Stangeriaceae (*Stangeria*), and Zamiaceae (*Bowenia, Ceratozamia, Dioon, Encephalartos, Lepidozamia, Macrozamia, Microcycas,* and *Zamia*).[1] Taxonomically, cycads are the lowest seed plants living now and are found only in tropical/subtropical areas of the world (Figure 1).[2] Each genus is more or less separated geographically from another. Most cycads are slow-growing and several species are unfortunately in danger of extinction. Cycads are dioecious, i.e., they produce separate male and female plants. The male forms narrow pine-like cones while the female develops seeds within larger cones. All cycads are terrestrial plants, with the exception of the epiphytic *Zamia pseudoparasitica* which grows on trees in the rain forest of Panama.

CYCAD CORALLOID ROOTS

In general, cycads have a trunk ranging in height from a few centimeters to more than 20 m, palm-like leaves, and a conspicuous tap root. From the tap root, three additional root structures may develop: lateral roots, coralloid roots, and apogeotropical roots. Particular attention has been given to the coralloid roots as they may harbor nitrogen-fixing cyanobacteria within a distinct cylindrical zone. As early as 1853, Schacht[3] described the occurrence of so-called "nebenwurzeln" in *Z. pumila*. The ability to form coralloid roots is coded for by genes in the cycad genome. When seeds of six different cycad genera from two families (Cycadaceae and Zamiaceae) were grown axenically, coralloid roots developed in all cases.[4] In some cycads the formation occurred in total darkness, while in others a light period was required. Moreover, a regeneration of coralloid roots has been observed in the megagametophytes of both *Macrozamia* and *Cycas* (Figure 2).[5,6] In all cycad species examined so far (about 50%), coralloid roots have been found (Table 1A). Even the artificial hybrid between *Ceratozamia hildae* and *C. microstrobila* and the epiphytic *Z. pseudoparasitica* produce these structures. Thus, the ability to produce such roots is likely to be a common feature of all cycad species. A collection of typical, dichotomously branched, cycad coralloid roots from the genera *Cycas, Encephalartos*, and *Stangeria* are shown in Figure 3.

In nature, the coralloid roots are usually found below the soil surface, although they grow apogeotrophically; in older plants they may reach the soil surface and be seen by the naked eye. Massive amounts of coralloid roots may, however, be seen at or above the soil surface in potted plants maintained in greenhouses.

THE CYANOBACTERIAL ZONE

On cross- or longitudinal-sectioning of a mature coralloid root, a pronounced dark blue-green band, the cyanobacterial zone, is evident (Figures 4a and b). Removal of the cyano-

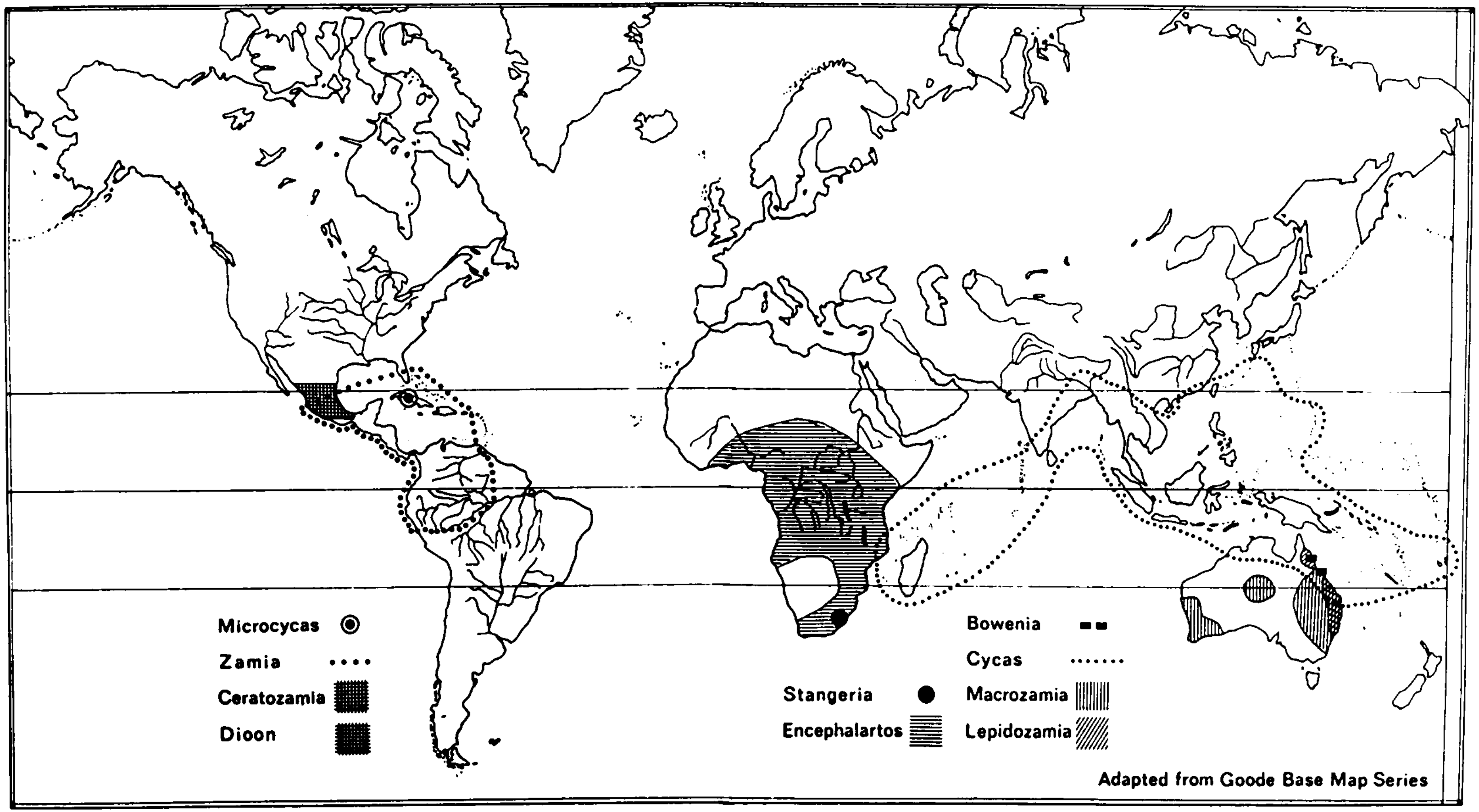
Microcycas ◉
Zamia ••••
Ceratozamia ▦
Dioon ■
Stangeria ●
Encephalartos ▤
Bowenia ▪▪
Cycas ......
Macrozamia ▥
Lepidozamia ▨
Adapted from Goode Base Map Series

FIGURE 1. World distribution of living cycad genera. (From Read, W. B. and Solt, M. L., *Lyonia*, 2, 37, 1986. With permission.)

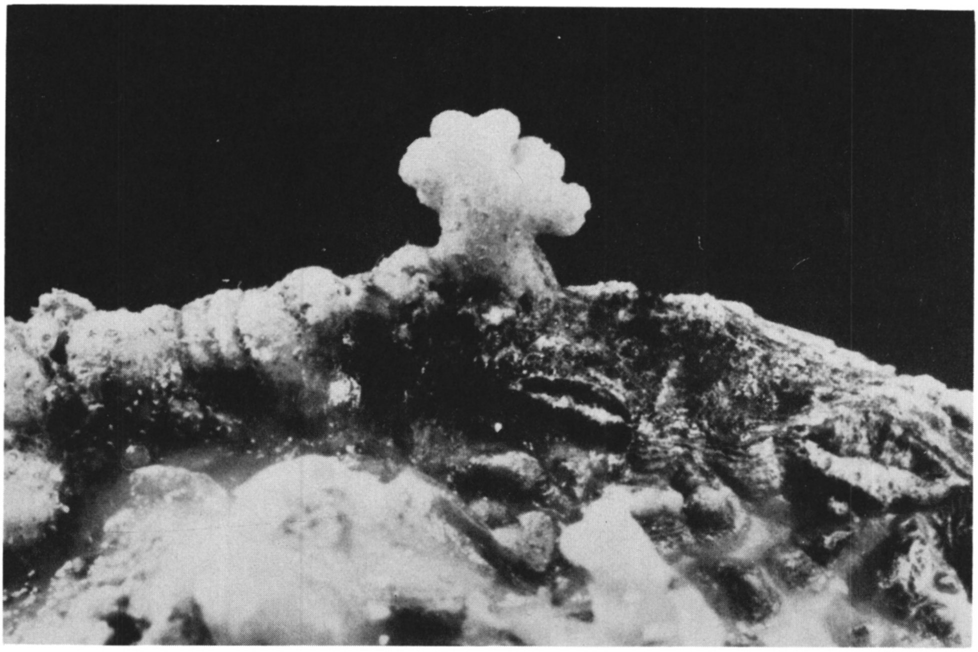

FIGURE 2. *Cycas revoluta* megagametophyte with formation of a coralloid root. (From DeLuca, P. and Sabato, S., *Plant Sci. Lett.*, 18, 27, 1980. With permission of Elsevier Scientific Publishers.)

TABLE 1
Occurrence of Coralloid Roots (A) and Their Ability to Fix Atmospheric Nitrogen (B) in Cycads

Cycad species	(A) Ref.	(B) Ref.
Cycadaceae		
Cycas angulata R. Br.	7	
C. circinalis L.	8, 9, 10	40, 45 (C_2H_2)
C. revoluta Thunb.	10, 11, 12	46, 47 (C_2H_2) 7, 22, 46 (^{15}N)
C. rumphii Miq.	10	
C. rumphii Miq. *seemannii* (A. Braun) Kanehira	13	
C. thouarsii Gaud.	14	14 (C_2H_2, ^{15}N)
Stangeriaceae		
Stangeria eriopus (Kunze) Nash	14	14 (C_2H_2, ^{15}N)
S. paradoxa Th. Moore	8	
S. schizodon Bull.	13	
Zamiaceae		
Bowenia serrulata Bull.	15	
B. spectabilis Hook	13	
Ceratozamia hildae Landry & Wilson	This paper[b]	
C. hildae × *microstrobila* artificial hybrid	This paper[c]	
C. mexicana Brongn.	9, 13	48 (^{15}N)
C. mexicana Brongn. *robusta* (Miq.) Dyer	16	
C. microstrobila Vovides & Rees	This paper[c]	
C. miqueliana Wendt.	This paper[d]	
C. muricata Miq.	3	
C. zaragozae Medellin-Leal	This paper[e]	
Dioon caputoi DeLuca, Sabato & Vazques-Torres	This paper[e]	
D. edule Lindley	8, 17	

TABLE 1 (continued)
Occurrence of Coralloid Roots (A) and Their Ability to Fix Atmospheric Nitrogen (B) in Cycads

Cycad species	(A) Ref.	(B) Ref.
D. mejiae Standley & Williams	This paper[f]	
D. purpusi Rose	This paper[c]	
D. spinolosum Dyer	13	
Encephalartos altensteinii Lehm.	7, 15, 18	14 (C_2H_2, ^{15}N)
E. arenarius Dyer	14	14 (C_2H_2, ^{15}N)
E. caffer (Thunb.) Lehm.	14	14 (C_2H_2, ^{15}N)
E. concinnus Dyer & Verdoorn	14	14 (C_2H_2, ^{15}N)
E. cupidus Dyer	14	14 (C_2H_2, ^{15}N)
E. cycadifolius (Jacq.) Lehm.	14	14 (C_2H_2, ^{15}N)
E. eugene-maraisii Verdoorn	14	14 (C_2H_2, ^{15}N)
E. ferox Bertol.	14	14 (C_2H_2, ^{15}N)
E. friderici-guilielmi Lehm.	14	14 (C_2H_2, ^{15}N)
E. ghellincki Lem.	14	14 (C_2H_2, ^{15}N)
E. gratus Prain	7	
E. heenanii Dyer	14	14 (C_2H_2, ^{15}N)
E. hildebrandii A. Br. & Bouché	14	14 (C_2H_2, ^{15}N)
E. horridus (Jacq.) Lehm.	14	14 (C_2H_2, ^{15}N)
E. humilis Verdoorn	14	14 (C_2H_2, ^{15}N)
E. inopinus Dyer	14	14 (C_2H_2, ^{15}N)
E. laevifolius Stapt & B. Davy	14	14 (C_2H_2, ^{15}N)
E. lanatus Stapf & B. Davy	14	14 (C_2H_2, ^{15}N)
E. latifrons Lehm.	14	14 (C_2H_2, ^{15}N)
E. lebomboensis Verdoorn	14	14 (C_2H_2, ^{15}N)
E. lehmannii Lehm.	14	14 (C_2H_2, ^{15}N)
E. longifolius (Jacq.) Lehm.	14	14 (C_2H_2, ^{15}N)
E. manikensis Gilliland	14	14 (C_2H_2, ^{15}N)
E. munchii Dyer & Verdoorn	14	14 (C_2H_2, ^{15}N)
E. natalensis Dyer & Verdoorn	7, 14	14 (C_2H_2, ^{15}N)
E. ngoyanus Verdoorn	14	14 (C_2H_2, ^{15}N)
E. paucidentatus Stapf & B. Davy	14	14 (C_2H_2, ^{15}N)
E. princeps Dyer	14	14 (C_2H_2, ^{15}N)
E. transvenosus Stapf & B. Davy	7, 14	14 (C_2H_2, ^{15}N)
E. trispinosus (Hook) Dyer	14	14 (C_2H_2, ^{15}N)
E. umbeluziensis Dyer	14	14 (C_2H_2, ^{15}N)
E. villosus Lem.	10, 14	14 (C_2H_2)
E. woodii Sander	14	14, 22 (^{15}N)
Lepidozamia peroffskyana Regel	7	14 (C_2H_2, ^{15}N)
Macrozamia communis (L.) Johnson	19, 20, 21	49 (^{15}N)
M. corallipes Hook	22	22 (C_2H_2, ^{15}N)
M. dennisoni F. Muell.	13	
M. fraseri Miq.	16	
M. lucida (L.) Johnson	7, 15	
M. macleyi Miq.	13	
M. miqueli (F. Muell.) A. DC.	7	
M. moorei F. Muell.	7	
M. pauli-guilielmi W. Hill & F. Muell.	7	
M. riedlei (Fisch ex Gaud.) Gardn.	7, 23, 24	23, 24 (C_2H_2) 7, 23 (^{15}N)
M. spiralis Miq.	25	
Zamia fairchildiana Gomez	This paper[b]	
Z. floridana A. DC.	26, 27	27, 47 (C_2H_2)
Z. furfuraceae L.	9	
Z. inermis Vovides, Rees & Vazques-Torres	This paper[d]	
Z. lindenii Regel	13	
Z. loddigesi Miq.	This paper[d]	

TABLE 1 (continued)
Occurrence of Coralloid Roots (A) and Their Ability to Fix Atmospheric Nitrogen (B) in Cycads

Cycad species	(A) Ref.	(B) Ref.
Z. longifolia Jacq.	25	
Z. pseudoparasitica Yates	This paper[b]	
Z. pumila L.	3	
Z. purpurea Vovides, Rees & Vazques-Torres	This paper[c]	
Z. skinneri Warcz.	28, 29, 30	29 (C_2H_2)

[a] Estimated using either acetylene reduction (C_2H_2) or ^{15}N-technique.
[b] Las Cruzes Botanic Garden, San Vito, Costa Rica.
[c] Xalapa Botanic Garden, Xalapa, Mexico.
[d] Field, Veracruz, Mexico.
[e] Field, Puebla, Mexico.
[f] Royal Botanic Gardens, Kew, U.K.

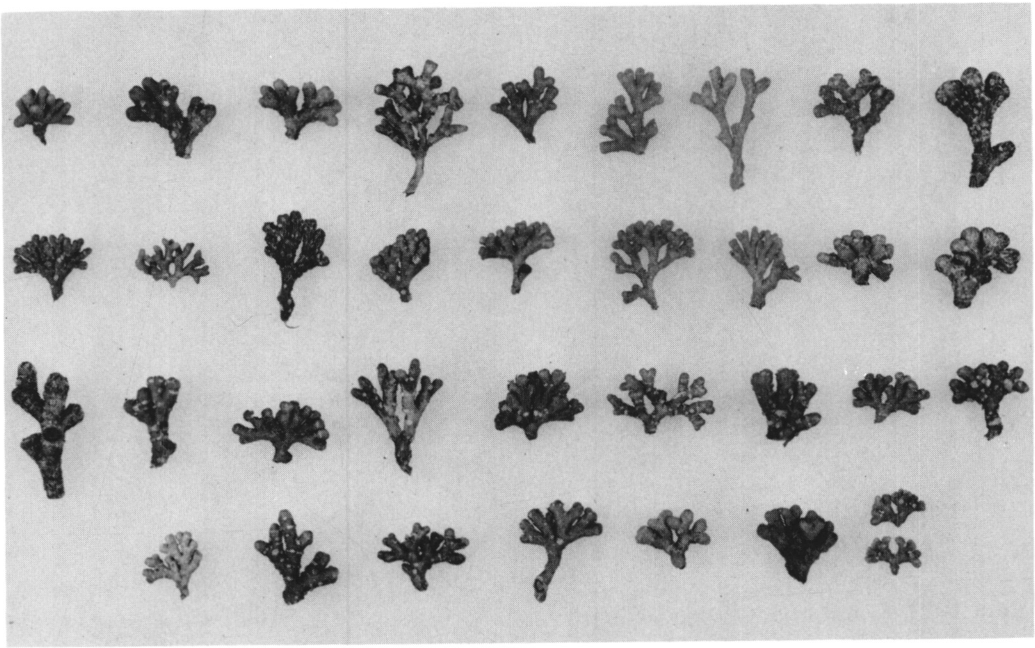

FIGURE 3. Cycad coralloid roots. From left to right, top row: *Cycas thouarsii, Encephalartos altensteinii, E. arenarium, E. caffer, E. concinnus, E. cupidus, E. cycadifolius, E. eugene-maraisii, E. ferox;* second row: *E. friderici-guiliemi, E. ghellinckii, E. heenanii, E. hildebrandtii, E. horridus, E. humilis, E. inopinus, E. laevifolius, E. lanatus;* third row: *E. latifrons, E. lebomboensis, E. lehmannii, E. longifolius, E. manikensis, E. munchii, E. natalensis, E. ngoyanus, E. paucidentatus;* bottom row: *E. princeps, E. transvenosus, E. trispinosus, E. umbeluziensis, E. villusos, E. woodii,* and *Stangeria eriopus.* (From Grobbelaar, N., Hatting, W., and Marshall, J., *S. Afr. J. Bot.*, 52, 467, 1986. With permission.)

bacterium and the mucilage from this zone (e.g., by washing) reveals a high number of elongated cycad cells transversing the cyanobacterial zone (Figure 4c). These cells, interconnecting two adjacent cortical layers, have been observed in several cycad genera/species.[9,21,28,31] It has been suggested that the elongated cells in *Z. skinneri* are specialized cells for the transfer of metabolites between the symbionts due to their considerably expanded

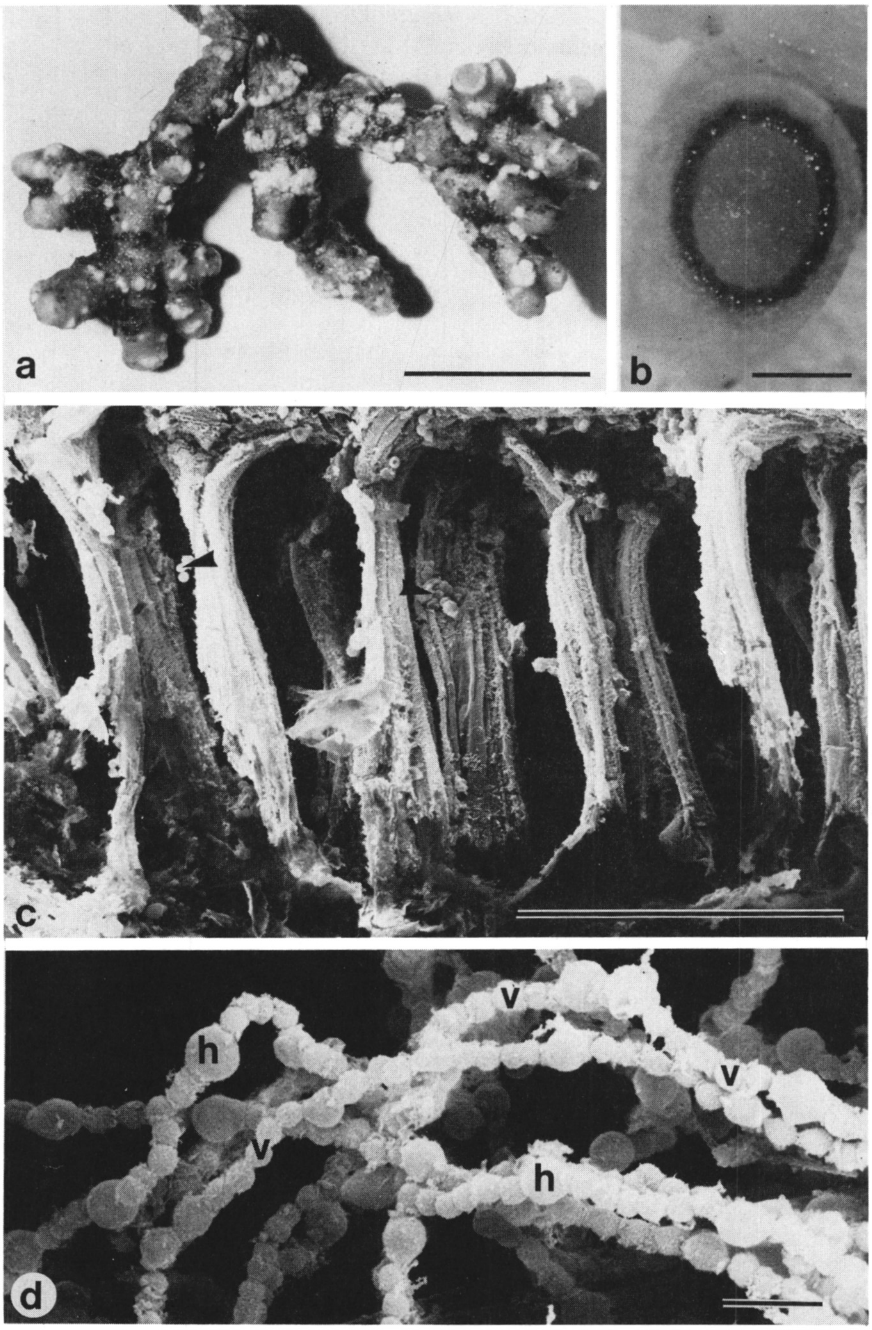

FIGURE 4. Coralloid root of *Zamia skinneri*. (a) Coralloid root, collected 10 cm below the soil surface. Bar = 5 mm. (b) Transverse section showing the cyanobacterial zone. Bar = 1 mm. (c) Scanning electron micrograph of the cyanobacterial zone, showing the elongated *Zamia* cells transversing the zone. Most of the cyanobacteria and mucilage is washed away. However, note a few remaining cyanobacterial cells (arrows). Bar = 100 μm. (d) Scanning electron micrograph of the cyanobacterial zone, showing cyanobacterial filaments with both heterocysts (h) and vegetative cells (v). Bar = 10 μm. (From Lindblad, P., Bergman, B., Hofsten, A. v., Hällbom, L., and Nylund, J. E., *New Phytol.*, 101, 707, 1985. With permission.)

cell walls and the abundance of mitochondria as well as amyloplasts.[28] The latter structures have also been found in the elongated cycad cells of *Cycas revoluta*[11,12] and *Encephalartos altensteinii*.[18] Structures showing similar characters have been described from other cyanobacterial-eukaryotic plant symbioses, e.g., *Azolla-Anabaena*, liverwort-*Nostoc*[28] (see also Chapters 3 and 4). In the majority of cases, filamentous heterocystous cyanobacteria occur intercellularly between the elongated cycad cells (Figure 4d).[7,9,11,12,20,28,31] There are, however, reports on intracellular cyanobacteria in coralloid roots of *C. revoluta*[11,32] and *Macrozamia communis*.[33]

CLASSIFICATION AND INFECTION

The cyanobacterium has been classified as *Nostoc* in 13 different cycad species within the 6 genera *Bowenia*,[15] *Cycas*,[10] *Dioon*,[10] *Encephalartos*,[10,34] *Macrozamia*,[10] and *Zamia*,[28] as *Anabaena* in *C. revoluta*,[11,12] and as both *Anabaena* and *Nostoc* in *M. communis*.[33] There is one report on the occurrence of *Calothrix* in a South African *Encephalartos* species.[34] it is also apparent that one particular cycad species may be infected by several cyanobacterial species.[34] Although it appears that only a limited number of cyanobacterial genera are capable of infecting cycads, the number of species, at least within the genus *Nostoc*, is considerable. Recently, chromosomal DNA prepared from cyanobacteria freshly isolated from coralloid roots of natural populations of *Ceratozamia mexicana, Dioon edule, Z. furfuraceae*, and *Z. skinneri* were compared. Restriction fragment length polymorphism analyses demonstrated that the cyanobacterial preparation showed differences in the sizes of kDNA fragments hybridizing to specific cyanobacterial probes, indicating that different cyanobacterial species and/or strains were in the symbiotic associations.[35] Moreover, the complexity of restriction patterns showed that a mixture of *Nostoc* strains can associate with a single cycad species, although a single cyanobacterial strain can predominate in the root of a single cycad plant.[35]

The fact that only a few heterocystous cyanobacterial genera are observed in coralloid roots of cycads suggests involvement of specific recognition signal(s) in establishment of such symbioses. It has been suggested that proteinaceous structures referred to as fimbriae are involved not only in motility of cyanobacteria but also in the specificity of the *Nostoc-Peltigera* symbiosis.[36] Interestingly, numerous fimbriae were detected on some cycad isolates, i.e., *Nostoc* 7422 (originally isolated from *Cycas*), *Nostoc* 73102 (*Macrozamia*), and *Nostoc* sp. (*Zamia*) (Figure 5). However, fimbriae are also present on free-living cyanobacteria, e.g., *Anabaena cylindrica, A. variabilis*, and *Nostoc muscorum*.[36a] The exact function of cyanobacterial fimbriae is not known, but an involvement in the early adherence and recognition cannot be ruled out.

So far, the infection process has been studied in only one cycad species, *M. communis*. The cyanobacterial filaments entered through a break in the dermal layer, then followed a continuous intercellular cortical channel into the cyanobacterial zone where the symbiosis was established (Figure 6).[20] Intracellular migration was also suggested as a likely pathway for the filaments into the cyanobacterial zone.[20] For discussion of earlier hypotheses regarding the mode of entry, see work by Nathanielsz and Staff.[20] Koch's postulate (i.e., separation and reunion of the organisms involved in the symbiosis) has not been fulfilled for the cycad-cyanobacterial symbiosis. It should be pointed out that very few studies have been conducted and that both the cycad and the symbiotic cyanobacteria may easily be grown as two separate organisms.

THE CYANOBIONT

The *Nostoc* filaments differentiate into vegetative cells, heterocysts, and occasionally akinetes (spores). Most striking is the intense blue-green color of the cyanobacterial zone,

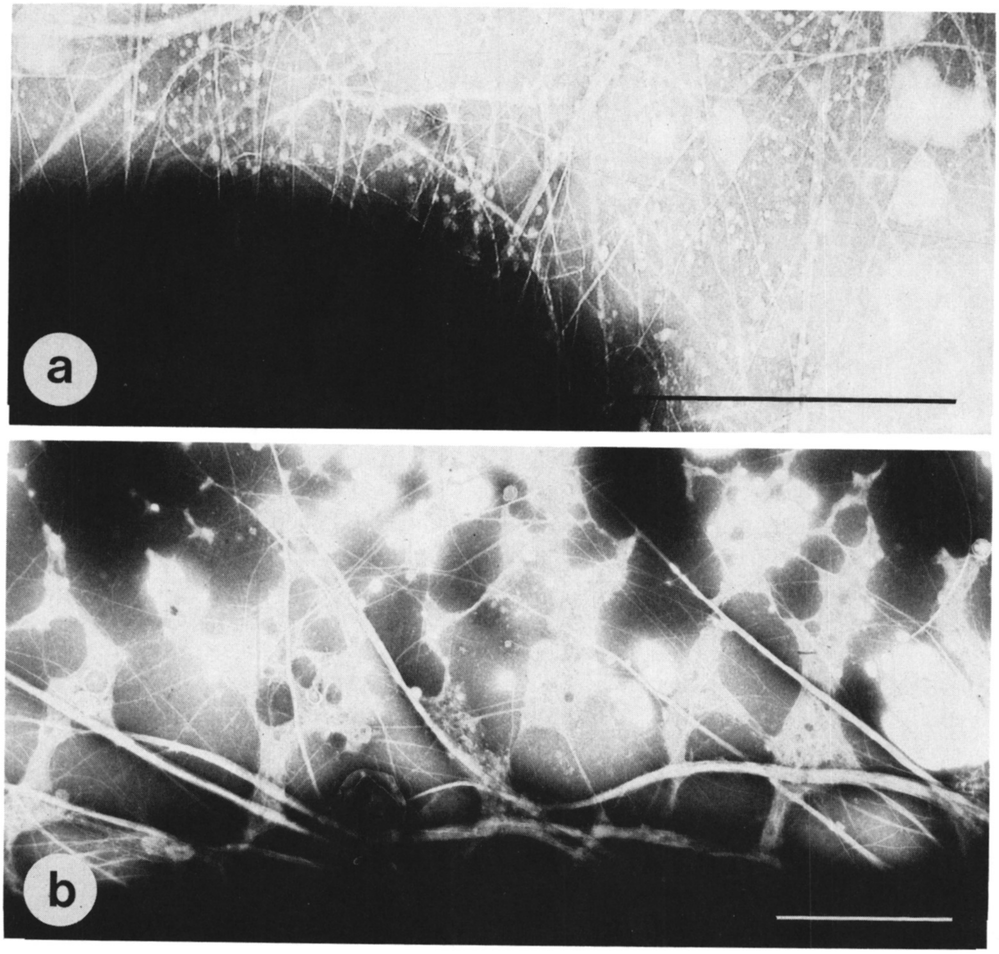

FIGURE 5. Negative staining of cyanobacterial strains originally isolated from cycads. Numerous fimbriae are present on both strains. (a) *Nostoc* 7422 (PCC 7422), isolated from *Cycas*. (b) *Nostoc* sp., isolated from *Zamia floridana*. Method as described in Reference 37. Bar = 1 μm.

which is due to the presence of chlorophyll *a* and phycobiliproteins in the vegetative cells. Structurally, the vegetative cells of the cycad cyanobiont examined so far show few modifications compared to its free-living isolates. The photosynthetic apparatus in the cyanobiont of *Z. skinneri* appears to be fully developed.[28] The vegetative cells contain thylakoids, with distinct phycobilisomes harboring the light-harvesting phycobiliproteins, as well as carboxysomes, the storage bodies containing the CO_2-fixing enzyme ribulose 1,5-bisphosphate carboxylase (Figure 7a).[28] Phycobilisomes are also present in the cyanobiont of the epiphytic cycad *Z. pseudoparasitica* (Figure 7b). By using an immunocytochemical technique, it was recently shown that the phycobiliprotein phycoerythrin is localized on thylakoid membranes in vegetative cells and absent from the heterocysts (both in the cyanobiont of *Cycas revoluta* and in its free-living isolate *Nostoc* 7422).[38] However, in ultrastructural studies, phycobilisomes have been reported to be absent from the cyanobiont of *M. communis*.[19] The cyanobionts of *Zamia* also contain both nitrogen (cyanophycin granules) and carbon (glycogen granules) reserve structures and retain their main photosynthetic pigment, chlorophyll *a*.[28] Polyphosphate granules have never been observed in the cyanobiont of *Z. skinneri*.[28] A high frequency of heterocysts has been described in the cyanobiont of several cycad genera/species.[10,12,18,29,39,40] Not only is there an increase in the heterocyst frequency, there is

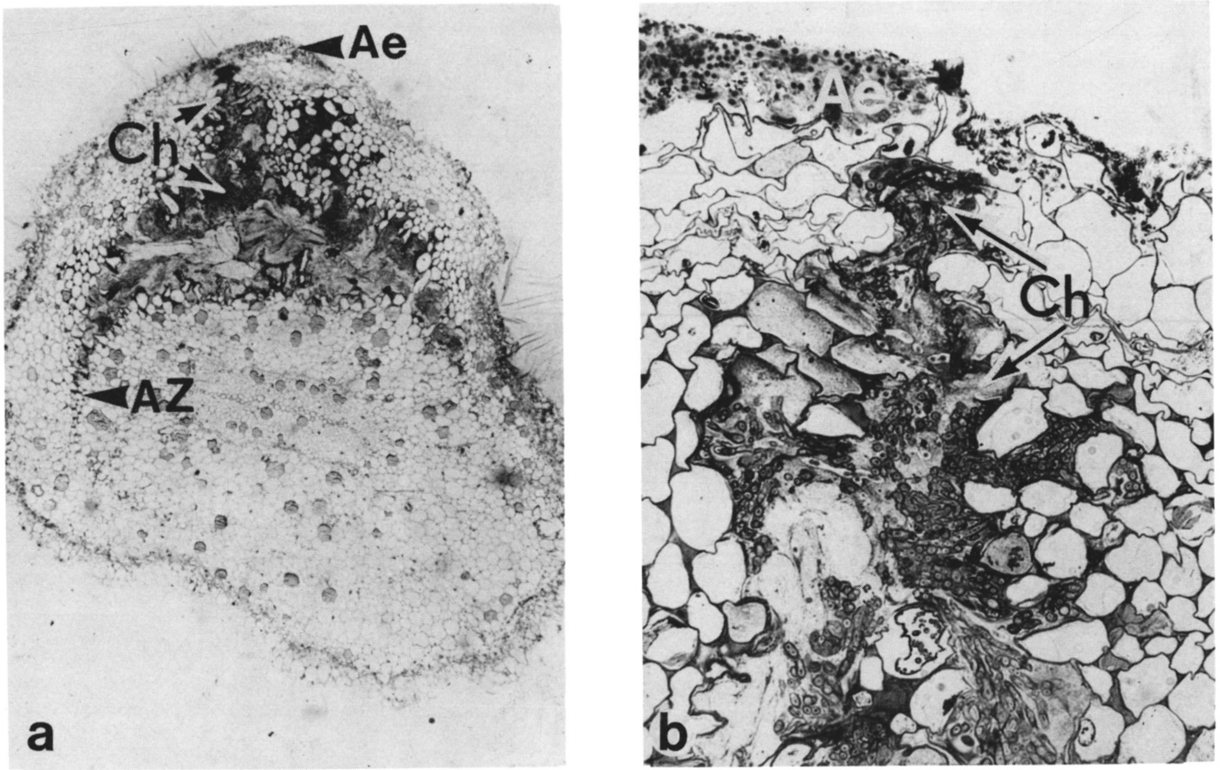

FIGURE 6. Entry of cyanobacteria into an aerial root of *Macrozamia communis*. (a) A transverse section through the root showing the entry of the cyanobacteria from the external mass into the cyanobacterial zone via a cortical channel. (Magnification × 30.) (b) The cortical channel at higher magnification. (Magnification × 140.) (From Nathanielsz, C. P. and Staff, I. A., *Am. J. Bot.*, 62, 232, 1975. With permission.)

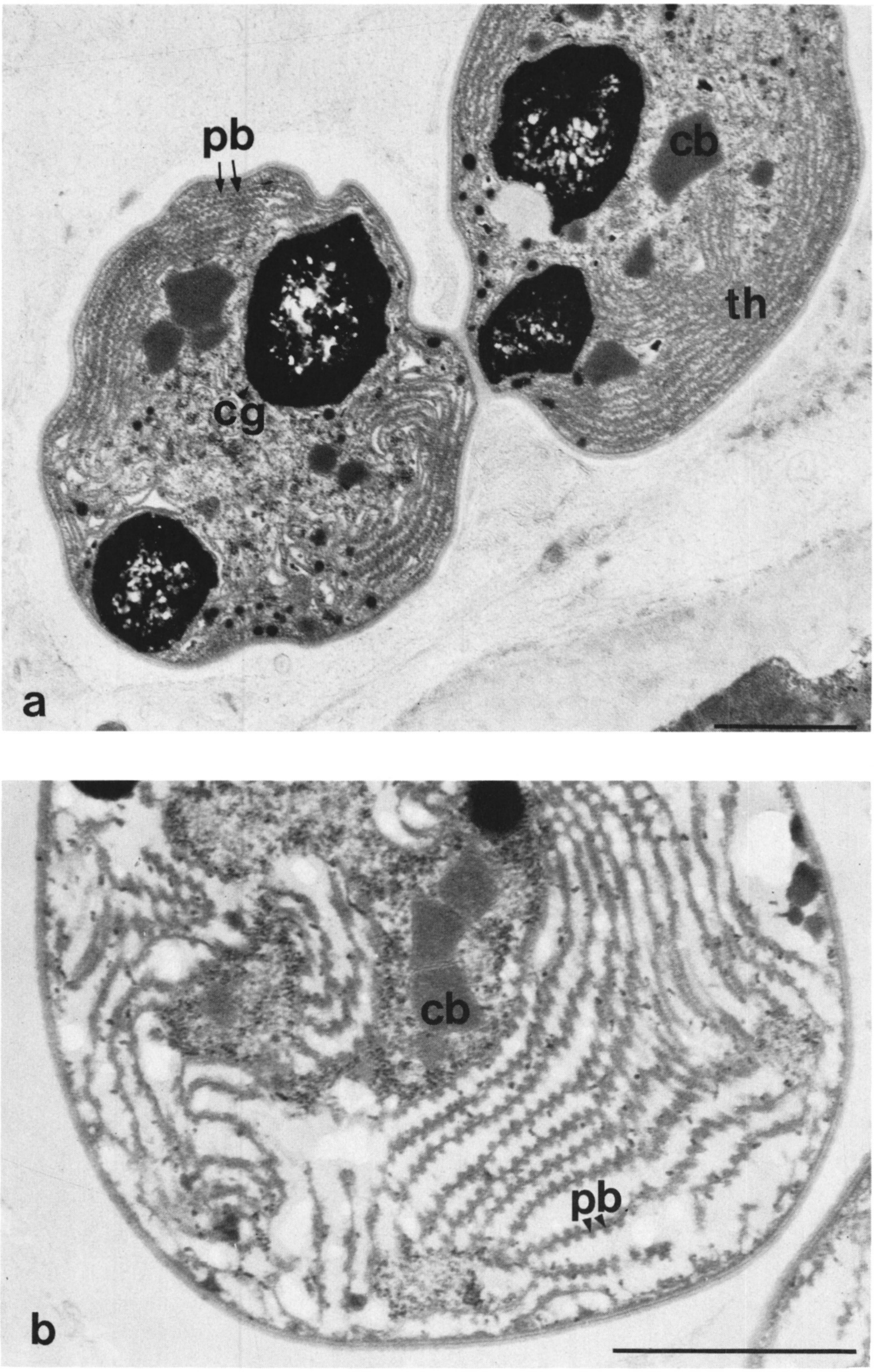

FIGURE 7. Cyanobacterial vegetative cells in coralloid roots of (a) *Zamia skinneri* and (b) *Z. pseudoparasitica*. Thylakoids (th) with distinct phycobilisomes (pb), carboxysomes (cb), and cyanophycin granules (cg) are present. Bar = 1 μm. ([a] From Lindblad, P., Bergman, B., Hofsten, A. v., Hällbom, L., and Nylund, J. E., *New Phytol.*, 101, 707, 1985. With permission.)

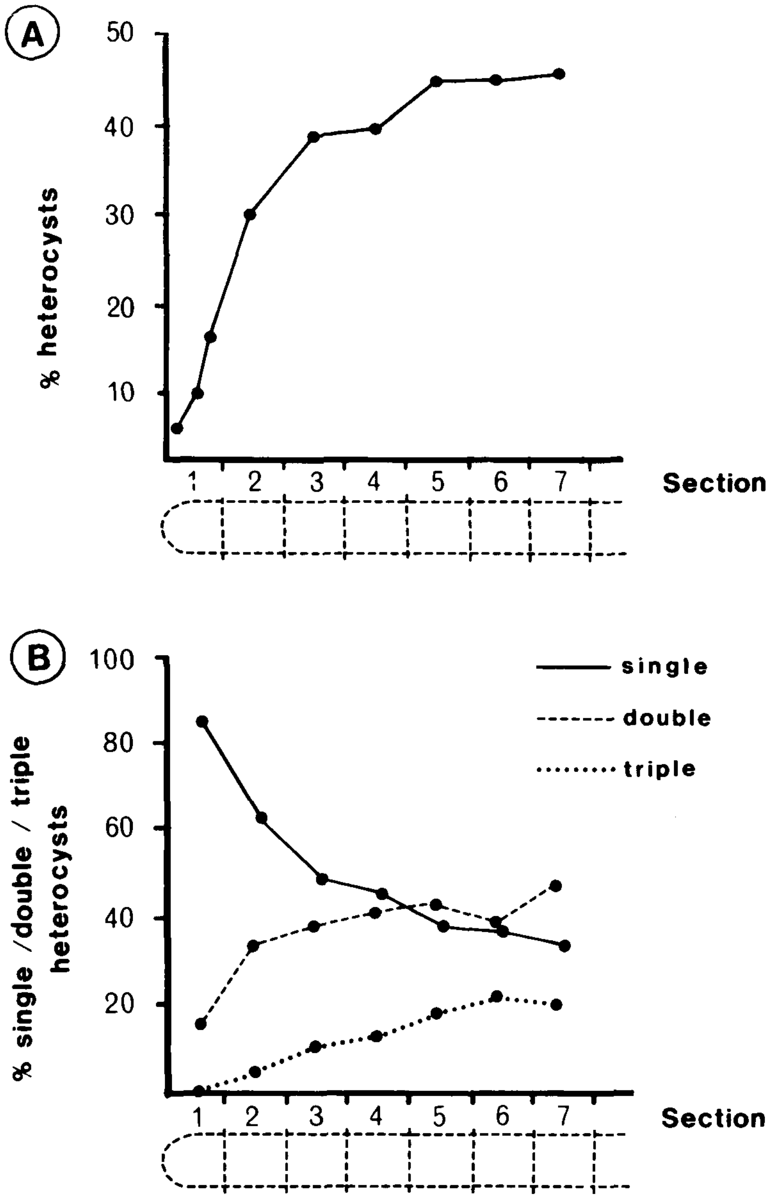

FIGURE 8. Heterocyst frequencies in different sections, each 1 to 2 mm, of a *Zamia skinneri* coralloid root. (A) Total heterocyst frequency. (B) Percentage single, double, and triple heterocysts out of the total number heterocysts. (From Lindblad, P., Hällböm, L., and Bergman, B., *Symbiosis*, 1, 19, 1985. With permission.)

also a change in the heterocyst spacing pattern. Moreover, a gradient in the heterocyst frequency, being low at the growing tip and higher in the older parts of the roots, is apparently a common phenomenon. For instance, a heterocyst frequency of 15 to 30% was found in the midregion of coralloid roots when 12 different cycad species, within 4 genera, were investigated.[10] A detailed examination of successive sections of coralloid roots of *Z. skinneri* revealed a dramatic increase in the heterocyst frequency, from 5 to 6% in the growing tip to about 46% in the basal, older parts (Figure 8A).[29,30] In the very tip of coralloid roots of *C. revoluta* a heterocyst frequency of 5 to 7% was also found,[30] a frequency similar to that observed in free-living nitrogen-fixing cyanobacteria. It was also apparent that, from being

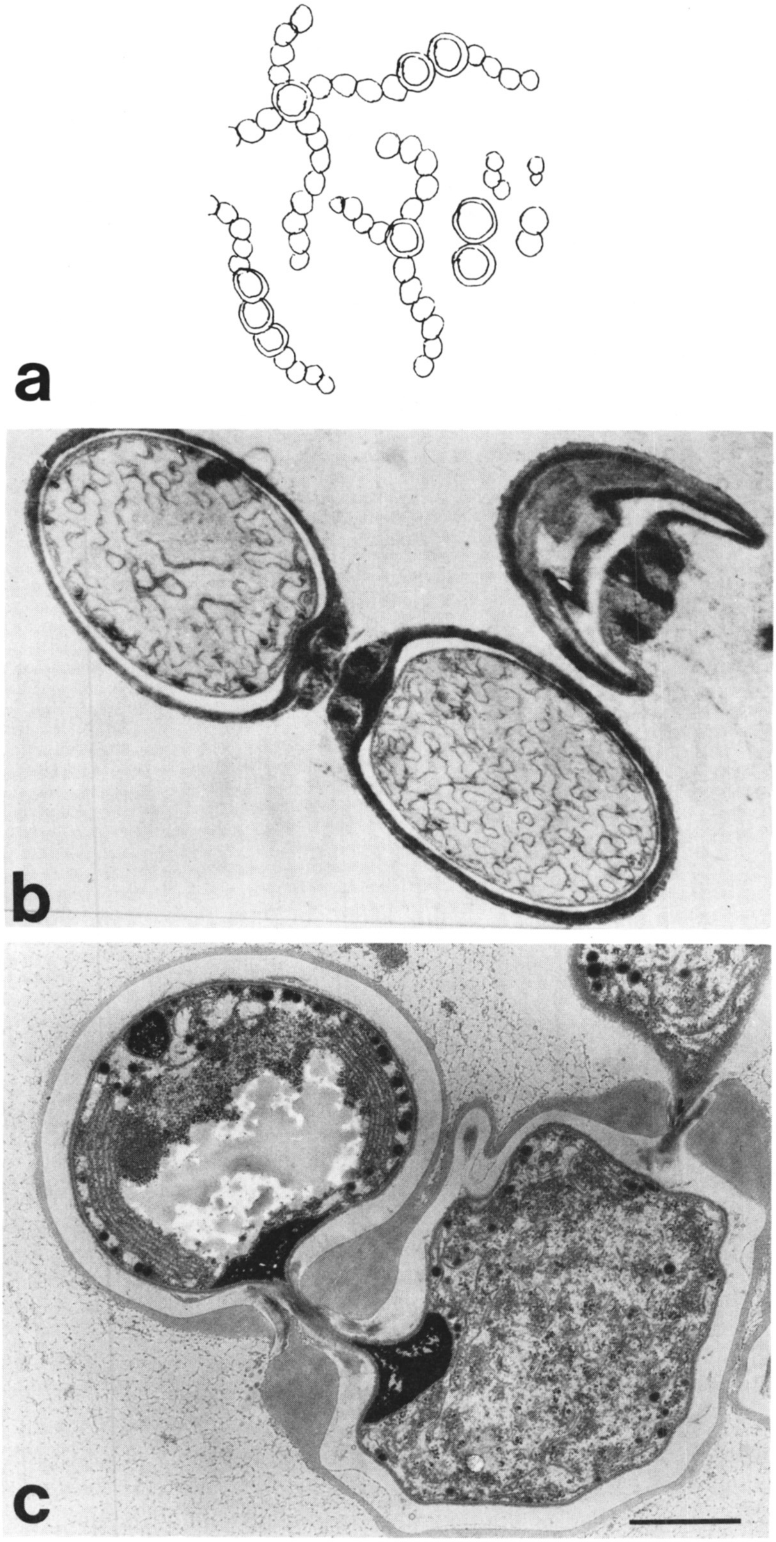

FIGURE 9.

mainly single at the growing tip, the heterocysts occur in multiples (double to quadruple) as the root ages. For instance, in *Z. skinneri* the relative amount of single heterocysts decreased from 86 to 31%. Simultaneously, double heterocysts increased from 14 to 47% and triple from 0 to 20% (Figure 8B). Even quadruple heterocysts were observed.[29] As early as 1894, Schneider[41] published drawings depicting what may be double and triple heterocysts in *Nostoc* filaments of *Cycas* (Figure 9a). Transmission electron micrographs of double heterocysts of *C. revoluta*[42,43] and *Z. skinneri*[28] are shown in Figure 9b and 9c, respectively. Multiple heterocysts are very rarely observed in free-living nitrogen-fixing cyanobacteria. Interestingly, addition of glucose or sucrose to *N. linckia* cultures triggers an increased frequency of joined heterocysts.[44] Akinetes are rarely found in the cyanobacterial zone, but have nevertheless been reported to occur in four different cycads: *C. revoluta,*[12] *D. edule,*[17] *M. communis,*[19] and *Z. skinneri.*[28]

NITROGEN FIXATION AND OCCURRENCE OF NITROGENASE

The ability to fix nitrogen has been reported in about 50 of the 150 cycad species described (Table 1B). Both the acetylene reduction technique and measurement of $^{15}N_2$-incorporation have been used to study nitrogen fixation. In all cases, active fixation of nitrogen was found. To our knowledge there is no report stating that infected coralloid roots are incapable of fixing nitrogen. This, together with the fact that the cyanobiont is always classified as a heterosystous cyanobacterium (*Nostoc, Anabaena, Calothrix*; see above), suggests that nitrogen fixation generally occurs in cycads. This may be exemplified by a recent study stating that in the 33 *Encephalartos* species examined which form coralloid roots, all were infected with cyanobacteria and all fixed considerable amounts of nitrogen.[14] When symbiotic and free-living cyanobacteria of *C. circinalis* were compared, nitrogenase activity (C_2H_2 reduction) was found to be approximately seven times higher in the symbiotic stage of the cyanobiont.[40] Our own studies using the cyanobiont freshly isolated from *Z. skinneri* and *C. revoluta* showed nitrogenase activity comparable to that of free-living cyanobacteria when expressed on the basis of chlorophyll *a*.[29,30] Occasionally, the nitrogenase activity of the growing tip did exceed those noted in free-living species. In coralloid roots of both *Zamia* and *Cycas*, there is always a distinct gradient in the nitrogenase activity, being highest at the root tip and considerably reduced at the basal end (Figure 10).[29,30] Curiously, the total heterocyst frequency showed an almost inverse pattern, being low at the tip while up to 45% in older parts of the root.[29,30] This finding emphasizes the fact that a high frequency of heterocysts does not mean that the nitrogenase activity is high. In *Z. skinneri,* however, there is a good general correlation between the percentage of single heterocysts and the nitrogenase activity,[30] indicating that the majority of the multiple heterocysts may be nonfunctional. The possibility of nitrogenase being synthesized in the vegetative cells of the cyanobiont is not excluded, in particular as a lowered oxygen tension may prevail in the cyanobacterial zone. In the absence of light, the photolysis of water and intracellular production of oxygen will be inhibited. Moreover, the zone is completely surrounded by respiratory cycad root tissue. Nonetheless, experiments using antibodies directed towards the Fe-protein of the nitrogenase complex clearly demonstrate that nitrogenase (Fe-protein) is localized in the heterocysts only (Figure 11).[50] The subcellular distribution is comparable to that of heterocystous, free-living cyanobacteria. Even multiple (double and triple) heterocysts contain the Fe-protein, suggesting that the lowered nitrogenase

FIGURE 9. Double heterocysts in cycad coralloid roots. (a) Drawing of double and triple heterocysts in *Nostoc* isolated from *Cycas*. (From Schneider, A., *Bot. Gaz.*, 25, 1894. With permission.) (b) Transmission electron micrograph (TEM) of a double heterocyst in *Cycas revoluta*. Bar = 1 μm. (From Grilli, M., *G. Bot. Ital.*, 70, 460, 1963. With permission.) (c) TEM of a double heterocyst in *Zamia skinneri*. Bar = 1 μm. (From Lindblad, P., Bergman, B., Hofsten, A. V., Hällbom, L., and Nylund, J. E., *New Phytol.*, 101, 707, 1985. With permission.)

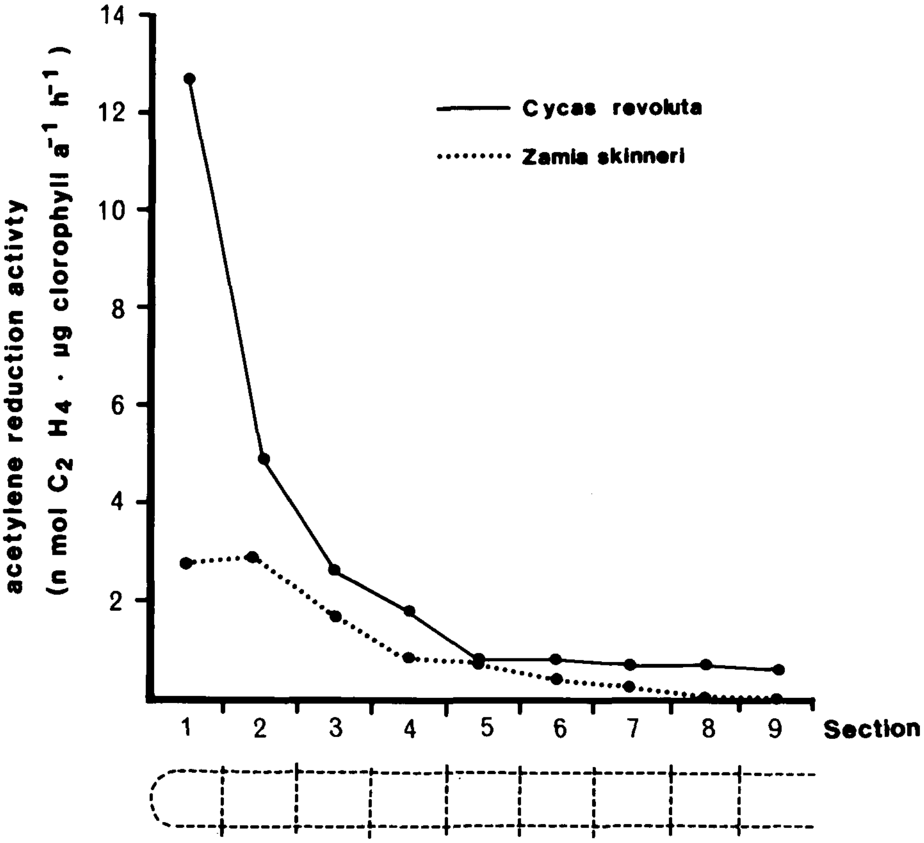

FIGURE 10. Nitrogen fixation (C_2H_2 reduction) in different sections of a coralloid root of *Cycas revoluta* and *Zamia skinneri*. The roots were cut into sections 1 to 2 mm thick and numbered 1 to 9, starting at the growing tip of the coralloid root. (Data from References 29 and 30.)

activity in the basal, older parts of the coralloid roots, containing a high proportion of multiple heterocysts, may be due to a hampered transport of important compounds from distant vegetative cells rather than to the absence of the nitrogenase protein per se.

HYDROGENASE

Nitrogenase also has the capacity to reduce hydrogen to H_2, a metabolically wasteful process that lowers the efficiency of the nitrogen fixation process. This loss may, however, be circumvented by an efficient uptake hydrogenase. In the cyanobiont of *C. circinalis* the uptake hydrogenase activity was undetectable, although present in the free-living isolate.[40,45] In the isolate, however, it was possible to abolish the activity of the uptake hydrogenase by adding glucose or placing the cells in the dark.[40] From these data it was concluded that the absence of hydrogenase activity in the cyanobiont may be explained by the fact that similar conditions prevail within the cyanobacterial zone of cycads.

FATE OF THE FIXED NITROGEN

The fate of the fixed nitrogen has recently generated considerable interest. As opposed to some other symbiotic cyanobacteria (e.g., in *Azolla* and in lichens), the cyanobionts of the cycads *Ceratozamia mexicana, Cycas revoluta*, and *Z. skinneri* all show *in vitro* activities

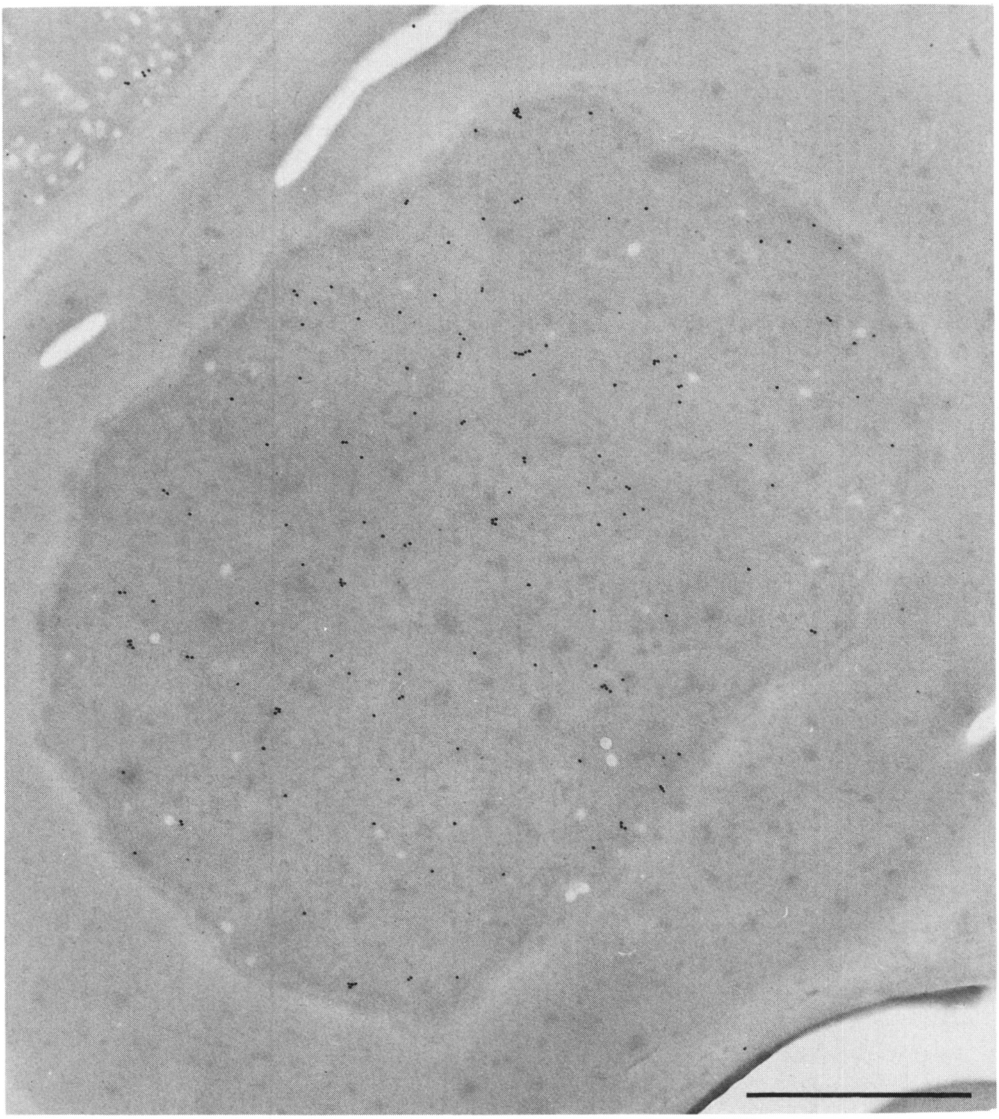

FIGURE 11. Immunocytochemical localization of Fe-protein of the nitrogenase complex in the cyanobiont of a *Cycas revoluta* heterocyst. Bar = 1 μm.

of the primary ammonium assimilating enzyme glutamine synthetase (GS) comparable to those of free-living nitrogen-fixing cyanobacteria (Table 2).[30] Moreover, the cellular levels, as well as the subcellular distribution, of the GS protein in the cyanobionts of both *C. revoluta* and *Z. skinneri* are comparable to those found in free-living isolates.[30] When estimating the relative cellular quantities of GS, GS protein level was noted to be 30 to 40% higher in heterocysts compared to that of vegetative cells (Table 3).[30] This seems reasonable, since the heterocysts are apparently the sole nitrogen-fixing loci.[50] As in the case of nitrogenase activity, there is a decline in the cyanobacterial GS activity towards basal parts of the roots of both *Cycas* and *Zamia*, although less drastic.[30] Ammonium-assimilating glutamate dehydrogenase (i.e., NADPH-dependent) is undetectable in the cyanobiont (Table 4).[45,51] In nitrogen-fixing cyanobacteria in general, glutamine is further converted to glutamate via a reaction catalyzed by the enzyme glutamate synthase (GOGAT). This enzyme

TABLE 2
In Vitro Glutamine Synthetase Activity in Symbiotic Cyanobacteria Freshly Isolated from Different Sections of Coralloid Roots and in Some Free-Living Cyanobacteria and a *Cycas* Isolate

Activity in Freshly Isolated Cyanobacteria

	Section of the coralloid root				
Cycad species	**1**	**2**	**3**	**4**	**5**
Ceratozamia mexicana	59	52	32	27	5
Cycas revoluta[a]	76	56	35	22	N.D.
Zamia skinneri[a]	60	74	59	24	N.D.

Activity in Free-Living Cyanobacteria and a *Cycas* Isolate

Anabaena cylindrica (CCAP 1403/2a)[b]	65 ± 10
Nostoc muscorum (CCAP 1453/12)[b]	55 ± 11
Nostoc 7422 (PCC 7422)[c]	62 ± 13

Note: N.D., not determined. Activity expressed as nanomoles NADH oxidized per minute per milligram protein.

[a] Data from Reference 30.

[b] Free-living cyanobacteria.

[c] Originally isolated from *Cycas*.

TABLE 3
Quantification of Relative Amounts of Glutamine Synthetase (GS) in the Cyanobionts of *Cycas revoluta* and *Zamia skinneri*

	Heterocyst	**Vegetative cell**	**Background**
Cycas revoluta			
GS-gold particles	100 ± 4	70 ± 2	10 ± 1
Cells/backgrounds counted	51	61	28
Gold particles counted	15,807	14,486	1,062
Zamia skinneri			
GS-gold particles	140 ± 6	101 ± 3	17 ± 1
Cells/backgrounds counted	40	77	40
Gold particles counted	17,199	16,444	1,898

Note: Thin sections of the growing tip of the coralloid roots were incubated with rabbit-anti-GS-antiserum, visualized using goat-anti-rabbit-IgG conjugated to 10 nm gold particles. Values given are number of gold particles per square micrometer of *Nostoc* cell, or background, and presented as means ± SE. All values within one species are significantly different at $p < 0.05$. Data from Lindblad, P. and Bergman, B., *Planta,* 169, 1, 1986.

shows considerable *in vitro* activity in the cyanobiont of *C. revoluta* (Table 4).[51] Only a few reports exist comparing *in vitro* enzyme activity in symbiotic cyanobacteria with that found in free-living isolates. Activity ranging from 30 to 60% lower has been noted for aspartate dehydrogenase, alanine dehydrogenase, and glutamate pyruvate aminotransferase in the cyanobiont of *C. circinalis* (Table 4).[45] However, glutamate oxaloacetic acid aminotransferase (GOT) showed higher *in vitro* activity in cyanobacteria freshly isolated from

TABLE 4
In Vitro* Activities of Nitrogen-Metabolizing Enzymes in the Cyanobionts of *Cycas circinalis* and in *C. revoluta

Enzymes[d]	*C. circinalis*[a]		*C. revoluta*[b]	
	Freshly isolated (symbiotic)	Free living (*Anabaena cycadeae*)	Freshly isolated (symbiotic)	Free living (*Nostoc* 7422)
GS (biosynthetic)	43 ± 2	60 ± 2	135.8	83.0
GOGAT	—	—	86.0	81.8
GDH				
(NADH-dependent)	—	—	17.6	7.4
(NADPH-dependent)	N.D.	30 ± 2	N.D.	N.D.
ADH (aminating)	10 ± 1	18 ± 1	—	—
AsDH (aminating)	7 ± 1	19 ± 1	—	—
GPT	27 ± 2	54 ± 3	3.7	N.D.
APT	—	—	27.0	22.7
GOT	22 ± 1	12 ± 1	70.2	20.6

Note: Abbreviations: GS = glutamine synthetase, GOGAT = glutamate synthase, GDH = glutamate dehydrogenase, ADH = alanine dehydrogenase, AsDH = aspartate dehydrogenase, GPT = glutamate pyruvate aminotransferase, APT = alanine dehydrogenase, and GOT = glutamate oxaloacetic acid aminotransferase. — = not determined and N.D. = not detectable. Activity expressed as nanomoles product formed per minute per milligram protein.

[a] Data from Perraju, B. T. V. V., Rai, A. N., Kumar, A. P., and Singh, H. N., *Symbiosis*, 1, 239, 1986.
[b] Data from Lindblad, P., Rai, A. N., and Bergman, B., *J. Gen. Microbiol.*, 133, 1695, 1987.

both *C. circinalis* and *C. revoluta* than those found in their respective free-living isolates (Table 4).[45,51] Using $^{15}N_2$, it has been clearly demonstrated that the fixed nitrogen is rapidly transferred from the cyanobiont to the cycad in *Cycas*[46] and *Macrozamia*.[49] By analyzing xylem sap from freshly detached coralloid roots of a number of cycads two strategies for the further processing of the fixed nitrogen were detected. Citrulline, glutamine, and to a lesser extent glutamic acid are the principal translocated N-solutes from coralloid roots to the rest of the cycad in *Macrozamia* (six spp.), *Lepidozamia* (one sp.), and *Encephalartos* (four spp.) and all members of the family Zamiaceae (Figure 12).[7] In contrast, glutamine and a smaller amount of glutamic acid, but not citrulline, were detected from the xylem sap of *Bowenia* (one species; Boweniaceae) and *Cycas* (two species; Cycadaceae) (Figure 12).[7] $^{15}N_2$ pulse-chase experiments, using *M. riedlei* coralloid roods, demonstrated a fast ^{15}N incorporation into glutamine and citrulline of both xylem sap and coralloid root tissues. However, labeling in glutamine preceded that in citrulline. This was followed by a decline in the ^{15}N label of glutamine in the coralloid root, while the label in citrulline progressively increased in both the coralloid tissues and the xylem sap. Similar experiments using $^{14}CO_2$ showed ^{14}C incorporation into aspartic acid and citrulline of the coralloid roots and into citrulline of xylem bleeding sap.[7] The ^{14}C of citrulline was restricted to the carbamoyl-carbon of the citrulline molecule. Similar studies on *C. revoluta* suggest glutamine to be the dominant product of $^{15}N_2$ fixation, while aspartic acid and alanine are the principal ^{14}C-labeled amino compounds: citrulline is undetectable.[7] The form in which fixed nitrogen is released by the cyanobiont is still not known. However, the fact that (unlike other cyanobacterial symbioses) the cyanobiont of *Cycas* possesses full GS-GOGAT activities suggests that the fixed nitrogen transferred from the cyanobiont may be a product of ammonia assimilation.[30,45,51] Furthermore, it was also found that the freshly isolated cyanobiont of *M. riedlei* synthesizes ^{14}C-citrulline when supplied with $^{14}CO_2$ and that the addition of ornithine stimulates citrulline synthesis.[52] However, cyanobacteria freshly isolated from *C. revoluta*, a symbiosis where no citrulline is synthesized *in situ*,[7] also produce ^{14}C-citrulline

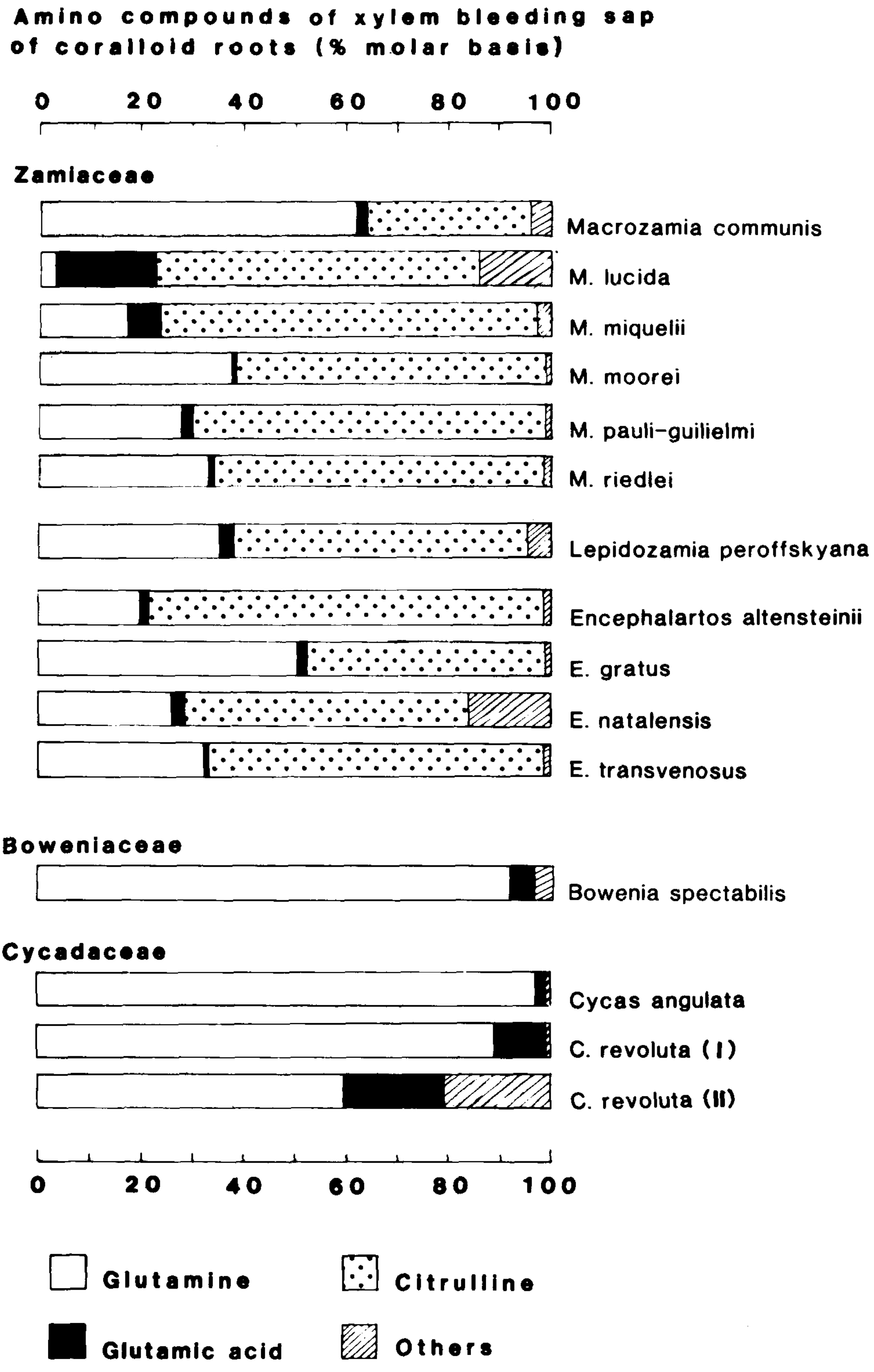

FIGURE 12. Nitrogenous solutes of xylem bleeding sap of coralloid roots of a range of cycad genera and species. Note the predominance of citrulline in members of the Zamiaceae but the absence of this compound in *Cycas* and *Bowenia*. (From Pate, J. S., Lindblad, P., and Atkins, C. A., *Planta*, 176, 461, 1988. With permission.)

TABLE 5
In Vitro Activities of Ribulose 1,5-Bisphosphate Carboxylase (RuBisCO) and Phosphoribulokinase (PRK), and Phosphoenolpyruvate (PEP) Carboxylase and Pyruvate Carboxylase in Symbiotic *Nostoc* Freshly Isolated from *Cycas revoluta* and in the Free-Living Isolate *Nostoc* 7422

	Symbiotic *Nostoc*	*Nostoc* 7422
RuBisCO	279.4	321.7
PRK	153.2	163.6
PEP carboxylase	<1.1	3.7
PEP carboxykinase	N.D.	<1.9
PEP carboxytransphosphorylase	<1.8	N.D.
Pyruvate carboxylase	3.5	4.2

Note: Activity expressed as nanomoles product formed per minute per milligram protein. N.D. = not detectable. Data from Reference 51.

when supplied with $^{14}CO_2$ together with ornithine.[52] Even nonsymbiotic *Macrozamia* organs (e.g., uninfected apogeotropical roots and lateral roots) synthesize ^{14}C-labeled citrulline when supplied with $^{14}CO_2$.[7] Thus, in *Macrozamia* coralloid roots both the cycad tissue[7] and the symbiotic cyanobacteria[52] have the capacity to synthesize citrulline. The precise location of the conversion of fixed N_2 into citrulline is presently being studied.

A biphasic uptake of ammonium (using the analog $CH_3NH_3^+$) in some free-living cyanobacteria has been described.[53] The first phase represents transport into the cells and the second phase is dependent upon sustained assimilation of ammonia via GS.[53] Using $^{14}CH_3NH_3^+$, an L-methionine-DL-sulfoximine-insensitive ammonium transport system (ATS) was found in the cyanobiont of *C. revoluta*.[54] However, in contrast to the situation in the free-living isolate,[55] the second phase was undetectable,[54] although the cyanobiont showed substantial *in vitro* GS activity.[30,54] Interestingly, microaerobiosis has been suggested to be involved in the repression of ATS in *Rhizobium meliloti*.[56]

CARBON FIXATION AND METABOLISM

The cyanobacteria in cycad coralloid roots are predominantly found below the soil surface. Therefore, a heterotrophic carbon metabolism in the cyanobiont has been suggested.[30,51] Fixed carbon may be provided for either by the photosynthetic host and/or by dark CO_2 fixation by the cyanobiont. As previously mentioned, the symbiotic cyanobacteria are living within a zone apparently filled with mucilage, the origin and chemical composition of which is unknown. There is no information available regarding transfer of fixed carbon from the cycad plant to the cyanobiont. In the cyanobiont of *C. revoluta* there is only low or undetectable *in vivo* CO_2 fixation.[51,52] However, both the primary carbon incorporating enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase, (RuBisCO) and the ribulose 1,5-bisphosphate-generating phosphoribulokinase show considerable *in vitro* activity in the cyanobiont as well as in the free-living isolate (Table 5).[51] Only low *in vitro* activities of other CO_2-fixing enzymes have been found (Table 5).[51] *Nostoc* cells directly isolated from *M. lucida* are capable of a heterotrophic growth in darkness.[57] Growth is significantly enhanced by addition of a mixture of amino acids and there was no significant difference in the ultrastructure of photoautotrophically and heterotrophically grown cells.[57] In view of these results, it seems plausible that carbon is derived from the host plant. Identification of the

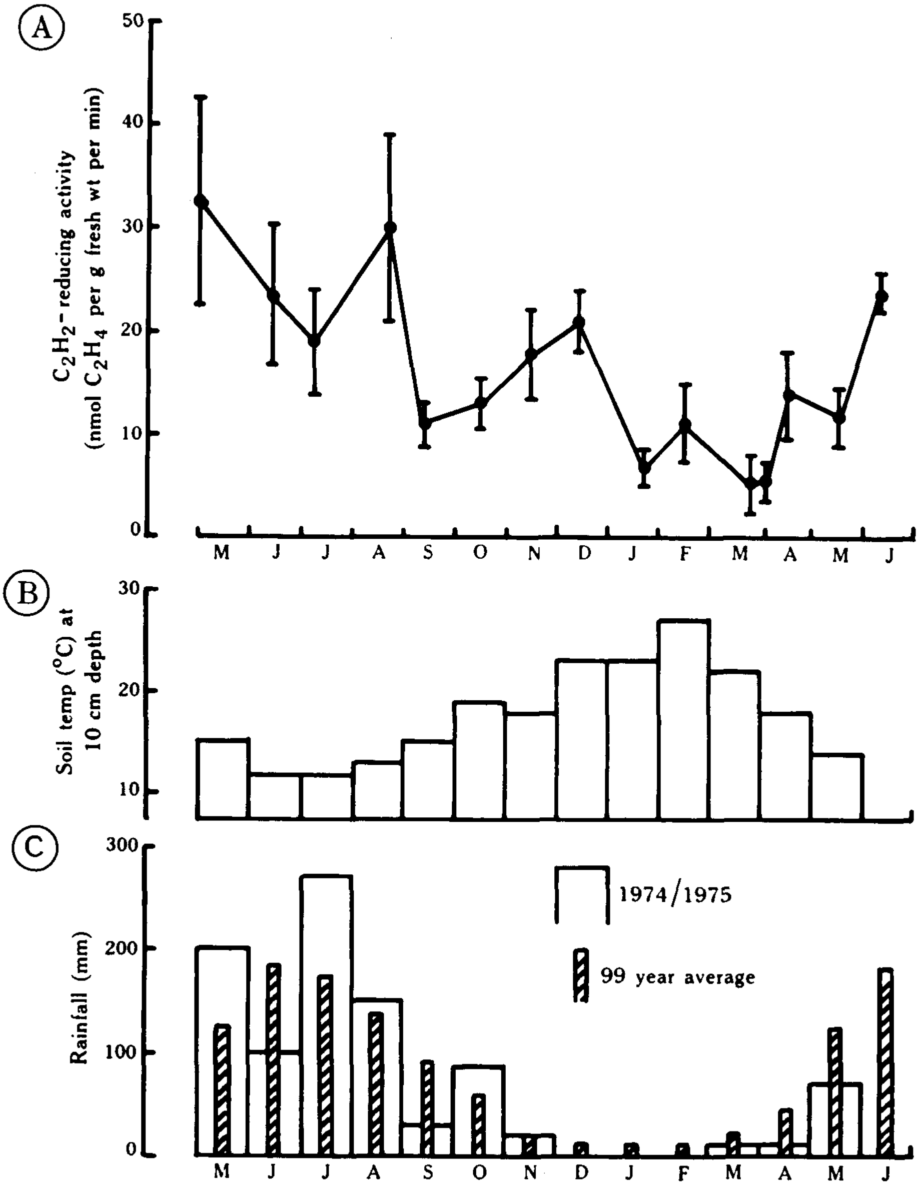

FIGURE 13. Nitrogen fixation by a natural population of *Macrozamia riedlei* in Australia, May 1974 to June 1975. (A) Seasonal variations in nitrogen fixation (C_2H_2 reduction) of field-grown *Macrozamia riedlei*. (B) Mean monthly soil temperature. (C) Total monthly rainfall, both for the studied period and the average values for the past 99 years. (From Halliday, J. and Pate, J. S., *Aust. J. Plant Physiol.*, 3, 349, 1976. With permission.)

carbon species transferred from the cycad to the symbiotic cyanobacteria merits further investigation.

When *in vitro* enzyme activities of the cyanobiont of *C. revoluta* and the free-living isolate were compared, increased activities of the two enzymes GOT (Table 4) and malate dehydrogenase (MDH) in the symbiotic stage were noted.[51] GOT and MDH may be involved in a malate-aspartate shuttle, transporting reducing equivalents from the cycad to the cyanobacterium as a substitute for those generated by light in free-living cyanobacteria.[51]

NITROGEN FIXATION IN THE FIELD

In several parts of Australia, *Macrozamia* species form a major part of the understory vegetation in the *Eucalyptus* forests. A nitrogen fixation capacity of approximately 19 kg N per hectare per year has been reported.[23] This leads to a doubling in the biomass of *Macrozamia* nitrogen in 8 to 11 years, or a doubling of the nitrogen content of the coralloid roots once every 8 weeks, taking into account the seasonal variations in the nitrogen fixation shown in Figure 13.[23] In a study of the effect of fire on nitrogen fixation in the same cycad species, a biomass doubling time of 6 years was found.[24] The nitrogen fixation decreased with time after the fire, from 8.4 kg N per hectare per year after 1.5 years to only 1.4 kg N per hectare per year after 7 years.[24] In both these studies,[23,24] it was concluded that nitrogen fixation through cycad coralloid roots contributes significantly to the nitrogen economy in their natural habitat.

USE OF CYCADS AND CYCAD TOXINS

Cycads have long been used as raw material for food production. Essentially, all parts of the cycad, including the stem, leaves, and seeds, are used. A major food product is cycad flour. Cycads contain an edible starch that can be ground into a dry meal. The seeds may be consumed raw or cooked and eaten as a biological vegetable. Thieret[58] provides a thorough review of the economic botany of cycads. However, cycads do not offer themselves for free. Most parts of the plant contain toxins, the biological effects of which include neurotoxicity, carcinogenicity, teratogenicity, and mutagenicity.[59] Fortunately, the toxins are water soluble and can therefore easily be washed away. The toxin cycasin has been found in all ten cycad genera[60] and macrozamin in *Bowenia, Cycas, Lepidozamia,* and *Macrozamia.*[61] For recent reviews of cycad azoxyglycosides and cycasin, the reader should consult works by Hoffman[62] and Morgan,[59] respectively.

ACKNOWLEDGMENTS

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REFERENCES

1. **Giddy, C.,** *Cycads of South Africa,* 2nd ed., C. Struik, Cape Town, 1984.
2. **Read, W. B. and Solt, M. L.,** Bibliography of the living cycads, *Lyonia,* 2, 37, 1986.
3. **Schacht, H.,** Beitrag zur Entwicklungs-Geschichte der Wurzel, *Flora,* 17, 257, 1853.
4. **Webb, D. T., Nevarez, M., and de Jesus, S.,** Further *in vitro* studies of light-induced nodulation in the Cycadales, *Environ. Exp. Bot.,* 24, 37, 1984.
5. **DeLuca, P. and Sabato, S.,** Regeneration of coralloid roots on cycad megagametophytes, *Plant Sci. Lett.,* 18, 27, 1980.
6. **DeLuca, P., Sabato, S., Balduzzi, A., and Nazzaro, R.,** Coralloid root regeneration on *Macrozamia* megagametophytes, *G. Bot. Ital.,* 114, 271, 1980.
7. **Pate, J. S., Lindblad, P., and Atkins, C. A.,** Pathways of assimilation and transfer of fixed nitrogen in coralloid roots of cycad-*Nostoc* symbioses, *Planta,* 176, 461, 1988.
8. **Milandasuta, B. E.,** Developmental anatomy of coralloid roots in cycads, *Am. J. Bot.,* 62, 468, 1975.
9. **Neumann, D.,** Ultrastrukturelle Untersuchungen zur Symbiose von Cyanophyceen mit Cycadeen (*Cycas circinalis* L., *Zamia furfuraceae* L.), *Biochem. Physiol. Pflanz.,* 171, 313, 1977.
10. **Grilli Caiola, M.,** On the phycobionts of cycad coralloid roots, *New Phytol.,* 85, 537, 1980.

11. **Obukowicz, M., Schaller, M., and Kennedy, G. S.,** Ultrastructure and phenolic histochemistry of the *Cycas revoluta-Anabaena* symbiosis, *New Phytol.*, 87, 751, 1981.
12. **Zhu, C.,** Fine structure of blue-green algae and the cells lined along the endophyte cavity in the coralloid roots of *Cycas* (in Chinese), *Acta Bot. Sin.*, 24, 109, 1982.
13. **Spratt, E. R.,** The root-nodules of the Cycadales, *Ann. Bot.*, 29, 619, 1915.
14. **Grobbelaar, N., Hatting, W., and Marshall, J.,** The occurrence of coralloid roots on the South African species of the Cycadales and their ability to fix nitrogen symbiotically, *S. Afr. J. Bot.*, 52, 467, 1986.
15. **Bowyer, J. W. and Skerman, V. B. D.,** Production of axenic cultures of soil-borne and endophytic blue-green algae, *J. Gen. Microbiol.*, 54, 299, 1968.
16. **Goebel, K.,** *Organographie der Pflanzen,* Part 3, 2nd ed., Gustav Fischer, Jena, 1923, 1284 pp.
17. **Grilli Caiola, M.,** Structural and ultrastructural aspects of blue-green algae growing in the coralloid roots of *Dioon edule* and in culture, *Phycos,* 14, 29, 1975.
18. **Grilli Caiola, M.,** A light and electron microscopic study of blue-green algae growing in the coralloid-roots of *Encephalartos altensteinii* and in culture, *Phycologia,* 14, 25, 1975.
19. **Grilli Caiola, M.,** A light and electron microscopic study of the blue-green algae living either in the coralloid roots of *Macrozamia communis* or in isolated culture, *G. Bot. Ital.*, 108, 161, 1974.
20. **Nathanielsz, C. P. and Staff, I. A.,** A mode of entry of blue-green algae into the apogeotropic roots of *Macrozamia communis, Am. J. Bot.*, 62, 232, 1975.
21. **Wittman, W., Bergersen, F. J., and Kennedy, G. S.,** The coralloid roots of *Macrozamia communis* L. Johnson, *Austr. J. Biol. Sci.*, 18, 1129, 1965.
22. **Watanabe, A. and Kiyohara, T.,** Symbiotic blue-green algae of lichens, liverworts and cycads, in *Studies on Microalgae & Photosynthetic Bacteria,* Japanese Society of Plant Physiologists, Tokyo, 1963, 189.
23. **Halliday, J. and Pate, J. S.,** Symbiotic nitrogen fixation by coralloid roots of the cycad *Macrozamia riedlei*: physiological characterization and ecological significance, *Aust. J. Plant Physiol.*, 3, 349, 1976.
24. **Grove, T. S., O'Connel, A. M., and Malajezek, N.,** Effects of fire on the growth, nutrient content, and rate of nitrogen fixation of the cycad *Macrozamia riedlei, Aust. J. Bot.*, 28, 271, 1980.
25. **Schaede, R.,** Über die Korallenwurzeln der Cycadeen und ihre Symbiose, *Planta,* 34, 98, 1944.
26. **Chaudhuri, M. and Akhtar, A. R.,** The coral-like roots of *Cycas revoluta, Cycas circinalis* and *Zamia floridana* and the alga inhabiting them, *J. Indian Bot. Soc.*, 10, 43, 1931.
27. **Lindblad, P.,** Diversion between C_2H_2-reduction and heterocyst frequency in a cycad root, in *Advances in Nitrogen Fixation Research,* Veeger, C. and Newton, W. E., Eds., Neihoff/Junk, The Hague, 1984, 511.
28. **Lindblad, P., Bergman, B., Hofsten, A. v., Hällbom, L., and Nylund, J. E.,** The cyanobacterium-*Zamia* symbiosis: an ultrastructural study, *New Phytol.*, 101, 707, 1985.
29. **Lindblad, P., Hällbom, L., and Bergman, B.,** The cyanobacterium-*Zamia* symbiosis: C_2H_2-reduction and heterocyst frequency, *Symbiosis,* 1, 19, 1985.
30. **Lindblad, P. and Bergman, B.,** Glutamine synthetase: activity and localization in cyanobacteria of the cycads *Cycas revoluta* and *Zamia skinneri, Planta,* 169, 1, 1986.
31. **Sprent, J. I.,** *The Biology of Nitrogen-Fixing Organisms,* McGraw-Hill, London, 1979.
32. **Grilli, M.,** Osservazioni sulle strutture e le infrastrutture delle cellule dei tubercoli radicali di *Cycas revoluta, Ann. Fac. Agrar.*, 3, 467, 1963.
33. **Nathanielsz, C. P. and Staff, I. A.,** On the occurrence of intracellular blue-green algae in cortical cells of the apogeotropic roots of *Macrozamia communis* L. Johnson, *Ann. Bot.*, 39, 363, 1975.
34. **Grobbelaar, N., Scott, W. E., Hattingh, W., and Marshall, J.,** The identification of the coralloid root endophytes of the southern African cycads and the ability of the isolates to fix dinitrogen, *S. Afr. J. Bot.*, 53, 111, 1987.
35. **Lindblad, P., Haselkorn, R., Bergman, B., and Nierzwicki-Bauer, S. A.,** Comparison of DNA restriction fragment lengths of *Nostoc* strains in and from cycads, *Arch. Microbiol.*, 152, 20, 1989.
36. **Dick, H. and Stewart, W. D. P.,** The occurrence of fimbriae on a N_2-fixing cyanobacterium which occurs in lichen symbiosis, *Arch. Microbiol.*, 124, 107, 1980.

36a. **Lindblad, P. and Bergman, B.,** unpublished data.

37. **Vaara, T.,** The outermost surface structures in chroococcacean cyanobacteria, *Can. J. Microbiol.*, 28, 929, 1982.
38. **Lindblad, P. and Bergman, B.,** Occurrence and localization of phycoerythrin in symbiotic *Nostoc* of *Cycas revoluta* and in the free-living isolated *Nostoc* 7422, *Plant Physiol.*, 89, 783, 1989.
39. **Spratt, E. R.,** Some observations in the life-history of *Anabaena cycadeae, Ann. Bot.*, 25, 369, 1911.
40. **Kumar, A. P., Perraju, B. T. V. V., and Singh, H. N.,** Carbon nutrition and the regulation of uptake hydrogenase activity in free-living and symbiotic *Anabaena cycadeae, New Phytol.*, 104, 115, 1986.
41. **Schneider, A.,** Mutualistic symbiosis of algae and bacteria with *Cycas revoluta, Bot. Gaz.*, 19, 25, 1894.
42. **Grilli, M.,** Osservazioni sulle infrastrutture di Anabaena cycadinae e delle cellule dei tubercoli radicali di *Cycas revoluta, G. Bot. Ital.*, 70, 460, 1963.
43. **Grilli, M.,** Richerche al microscopia elettronico sulle cianoficee viventi nei tubercoli radicali di *Cycas revoluta, Ann. Microbiol. Enzimol.*, 13, 109, 1963.

44. **Ladha, J. K. and Kumar, H. D.**, Response of a wild-type and non-nitrogen-fixing mutant of *Nostoc linckia* to different carbon sources, *New Phytol.*, 79, 299, 1977.
45. **Perraju, B. T. V. V., Rai, A. N., Kumar, A. P., and Singh, H. N.**, *Cycas circinalis-Anabaena cycadeae* symbiosis: photosynthesis and the enzymes of nitrogen and hydrogen metabolism in symbiotic and cultured *Anabaena cycadeae, Symbiosis*, 1, 239, 1986.
46. **Renaut, J., Sasson, A., Pearsson, H. W., and Stewart, W. D. P.**, Nitrogen-fixing algae in Morocco, in *Nitrogen Fixation by Free-Living Micro-organisms (IBP Handbook No. 6)*, Stewart, W. D. P., Ed., Cambridge University Press, Cambridge, 1975, 229.
47. **Stewart, W. D. P., Fitzgerald, G. P., and Burris, R. H.**, Acetylene reduction by nitrogen-fixing blue-green algae, *Arch. Microbiol.*, 62, 336, 1968.
48. **Bond, G.**, Fixation of nitrogen by higher plants other than legumes, *Annu. Rev. Plant Physiol.*, 18, 107, 1967.
49. **Bergersen, F. J., Kennedy, G. S., and Wittman, W.**, Nitrogen fixation in the coralloid roots of *Macrozamia communis* L. Johnson, *Aust. J. Biol. Sci.*, 18, 1135, 1965.
50. **Bergman, B., Lindblad, P., and Rai, A. N.**, Nitrogenase in free-living and symbiotic cyanobacteria: immunoelectron microscopic localization, *FEMS Microbiol. Lett.*, 35, 75, 1986.
51. **Lindblad, P., Rai, A. N., and Bergman, B.**, The *Cycas revoluta-Nostoc* symbiosis: enzyme activities in nitrogen and carbon metabolism in the cyanobiont, *J. Gen. Microbiol.*, 133, 1695, 1987.
52. **Lindblad, P., Atkins, C. A., and Pate, J. S.**, Unpublished data.
53. **Rai, A. N., Rowell, P., and Stewart, W. D. P.**, Evidence for an ammonium transport system in free-living and symbiotic cyanobacteria, *Arch. Microbiol.*, 137, 241, 1984.
54. **Rai, A. N., Lindblad, P., and Bergman, B.**, Absence of the glutamine-synthetase-linked methylammonium (ammonium) transport system in the cyanobiont of *Cycas*-cyanobacterial symbiosis, *Planta*, 169, 379, 1986.
55. **Singh, D. T., Rai, A. N., and Singh, H. N.**, Methylammonium (ammonium) uptake in a glutamine auxotroph of the cyanobacterium *Anabaena cycadeae, FEBS Lett.*, 186, 51, 1985.
56. **Pargent, W. and Kleiner, D.**, Characteristics and regulation of ammonium (methylammonium) transport in *Rhizobium meliloti, FEMS Microbiol. Lett.*, 30, 257, 1985.
57. **Hoare, D. S., Ingram, L. O., Thurston, E. L., and Walkup, R.**, Dark heterotrophic growth of an endophytic blue-green alga, *Arch. Microbiol.*, 78, 310, 1971.
58. **Thieret, J. W.**, Economic botany of the cycads, *Econ. Bot.*, 12, 3, 1958.
59. **Morgan, R. W. and Hoffmann, G. R.**, Cycasin and its mutagenic metabolites, *Mutat. Res.*, 114, 19, 1983.
60. **DeLuca, P., Moretti, A., Sabato, S., and Siniscalco Gigliano, G.**, The ubiquity of cycasin in cycads, *Phytochemistry*, 19, 2230, 1980.
61. **Moretti, A., Sabato, S., and Siniscalco Gigliano G.**, Distribution of macrozamin in Australasian cycads, *Phytochemistry*, 20, 1415, 1981.
62. **Hoffmann, G. R. and Morgan, R. W.**, Review: putative mutagens and carcinogens in foods. Cycad azoxyglycosides, *Env. Mutagen.*, 6, 103, 1984.

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Chapter 7

THE *NOSTOC-GUNNERA* ASSOCIATION

Howard T. Bonnett

INTRODUCTION

Although *Nostoc* species have established symbiotic relationships across generic lines in more primitive organisms such as lichens, mosses, and cycads, of all angiosperms, only *Gunnera* is known to establish a symbiotic relationship with *Nostoc*. While this genus is usually restricted to warm, moist habitats, its distribution is quite diverse, including New Zealand, Southeast Asia, Central America, and South America. Ten of the species grow in New Zealand, and *Nostoc* has been shown to occur in all of these.[1] In addition, work has been reported on *Gunnera macrophylla* in Indonesia,[2] *G. kaalensis* in Hawaii,[3] and *G. chilensis* growing in Germany.[4] Since no species has been described without *Nostoc*, all species of the genus probably grow in association with *Nostoc*.

An explanation for the unique ability of this genus to harbor *Nostoc* would not only be of interest in elaborating the basis for success of this symbiosis, but also could provide direction for experiments to introduce *Nostoc* to other angiosperm genera. Experiments of this type have been attempted over the past decade. Although cyanobacteria and cyanelles have been successfully introduced into plant protoplasts, no stable associations have been obtained. The current status of this work is summarized in the article by Gusev et al.[5] (also see Chapter 8).

Possible explanations for the success of the *Gunnera-Nostoc* association could involve the following *Gunnera* attributes: (1) hereditary material to make glands with unique features; (2) unusual structure of the *Gunnera* cell wall; (3) tolerance of the cells to cyanobacterial introduction and replication; (4) intracellular conditions favoring nitrogen fixation, such as low levels of fixed nitrogen, which would induce heterocyst differentiation and selection for retention of *Nostoc*; and (5) production of a favorable microenvironment for growth of *Nostoc* as an epiphyte on the apex.

Several aspects of this symbiosis have been reviewed in previously published volumes.[6,7] These should be consulted for historical details. This review summarizes our current understanding of the symbiosis and focuses on features which need additional work to enhance our understanding of the association.

DEVELOPMENT OF THE GLAND

Nostoc cells are plentiful in extracellular locations on the *Gunnera* plant (such as the mucus covering the shoot apex), but they colonize only internal portions of the plant where glands are produced. Thus, characteristics associated with the development of the gland are essential to the process of colonization which ultimately leads to an association of *Nostoc* cells within *Gunnera* cells. Based on anatomical studies of numerous investigators on several different species,[1,3,4,8-10] certain features of the gland are consistently found in the genus. These include an endogenous origin of the gland due to resumption of mitotic activity by one or more cells (Figure 1); an organization in which independent populations of cells form within the gland, each producing a multicellular, papillate extension towards the exterior (Figure 2); and a casting aside of the epidermal layer covering the papillae (Figure 3). Whatever the mode of entry of *Nostoc* cells into *Gunnera* cells, *Nostoc* cells must be unable to penetrate the epidermal cell layer. The pattern of development of the gland permits *Nostoc* cells to bypass the epidermal barrier.

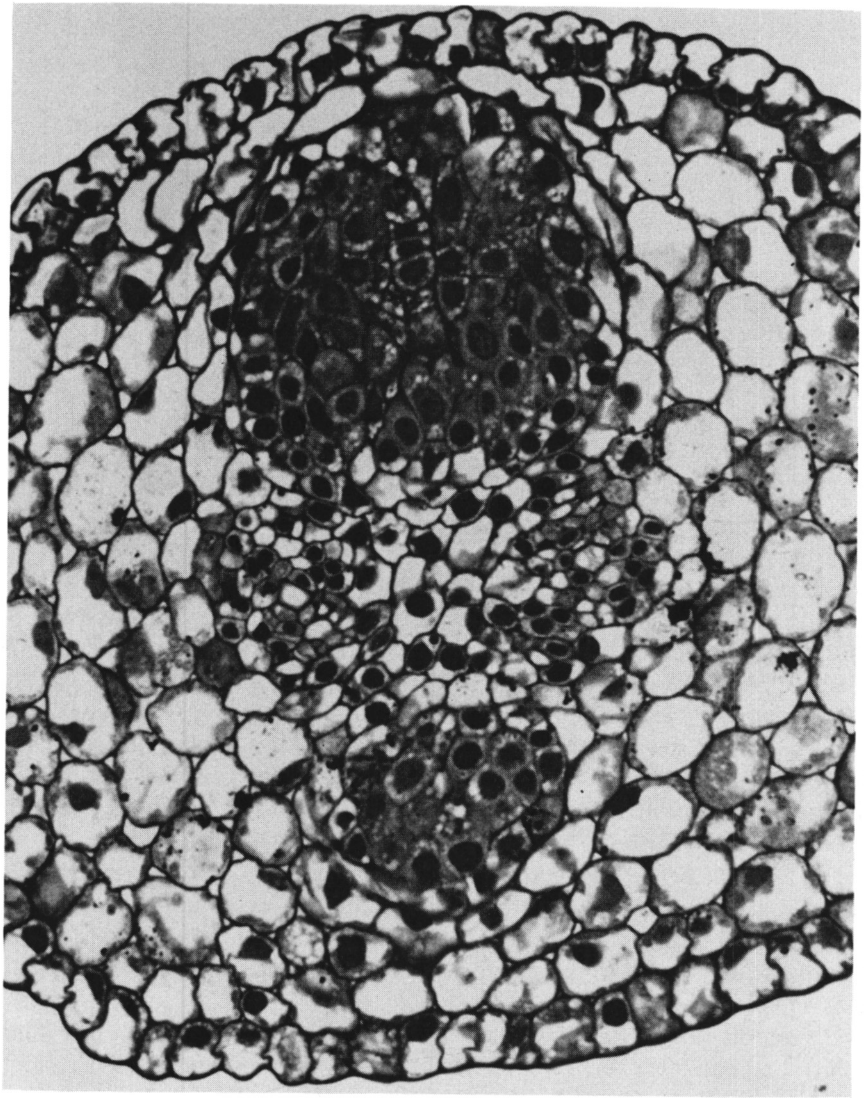

FIGURE 1. Cross section of a young seedling of *Gunnera chilensis*. Note that the two developing glands on the hypocotyl have originated in an endogenous position. (Magnification × 275.) (Photograph courtesy of E. Hoffmann.)

The papillate organization of the gland has an additional important consequence. It results in intercellular channels which lead to interior regions of the gland, particularly evident at the points where three papillae abut. The study by Hoffmann[10] has shown that the gland of *G. chilensis* typically possesses a central papilla with five to seven circumscribing papillae (Figure 4). Such an organization would produce four to six channel junctures where three papillae abut. Since mature, colonized glands frequently contain several separate clusters of cyanobacteria-containing cells,[1,2,6,11] each cluster could result from independently occurring entry of *Nostoc* at different channel junctures.

At the stage when the epidermis is cast off, most meristematic-appearing, thin-walled glandular cells are deeper in the gland. Because there have been no reports of glandular cells without cell walls, the assumption is that the mechanism of entry by *Nostoc* cells into *Gunnera* cells must involve penetration of the cell wall. The location of meristematic cells

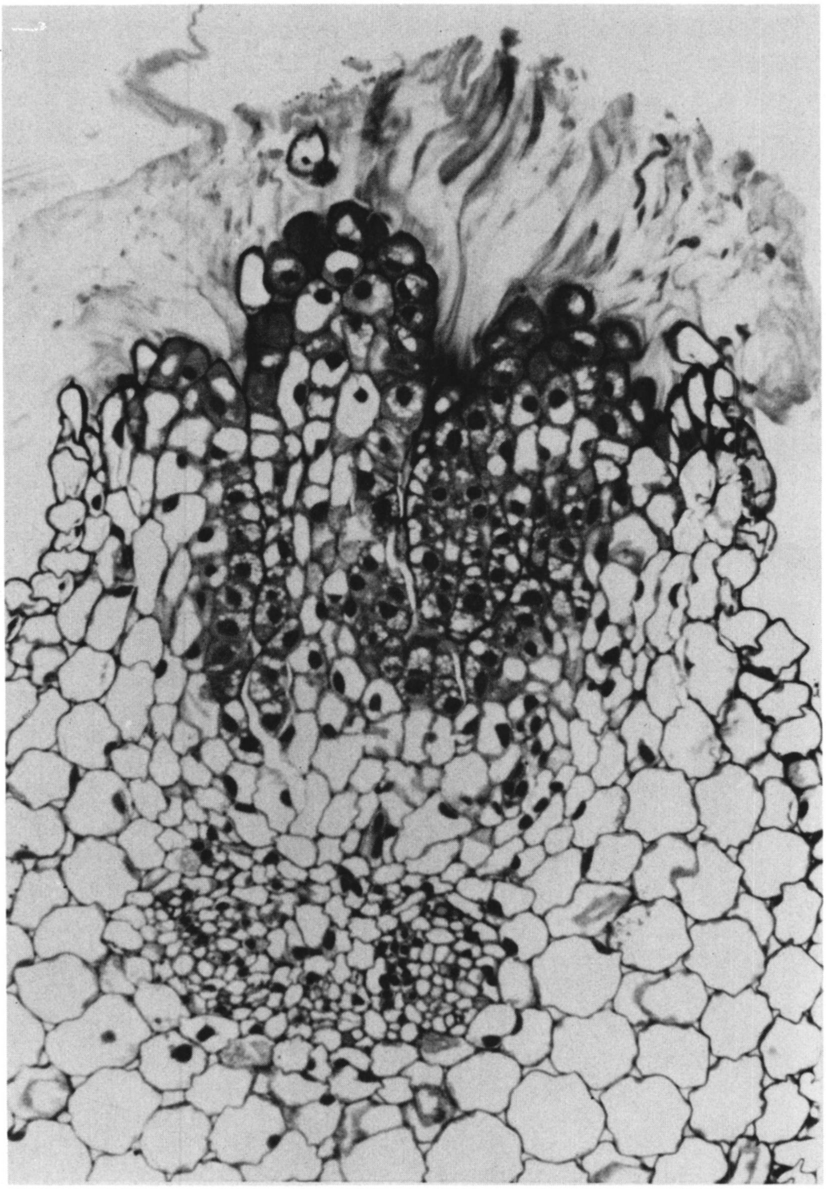

FIGURE 2. Light micrograph of a seedling gland 12 d after germination. Three papillae are shown in this section. Mucus covering the gland is readily visible. (Magnification × 160.) (Photograph courtesy of E. Hoffmann.)

at the base of the channels further supports a colonization pathway involving migration of *Nostoc* filaments into the gland via a mucus-filled channel juncture, and then penetration of the thinner-walled meristematic cells. The mobility of the *Nostoc* filaments, pushing against the mucus-lined confines of the channel, could provide the force to intrude to the cell interior.

COLONIZATION OF *GUNNERA* CELLS BY *NOSTOC* CELLS

No one who has studied this symbiosis with the electron microscope has achieved images purporting to show the transit of a *Gunnera* cell by *Nostoc* cells or filaments.[1,3,4] Therefore,

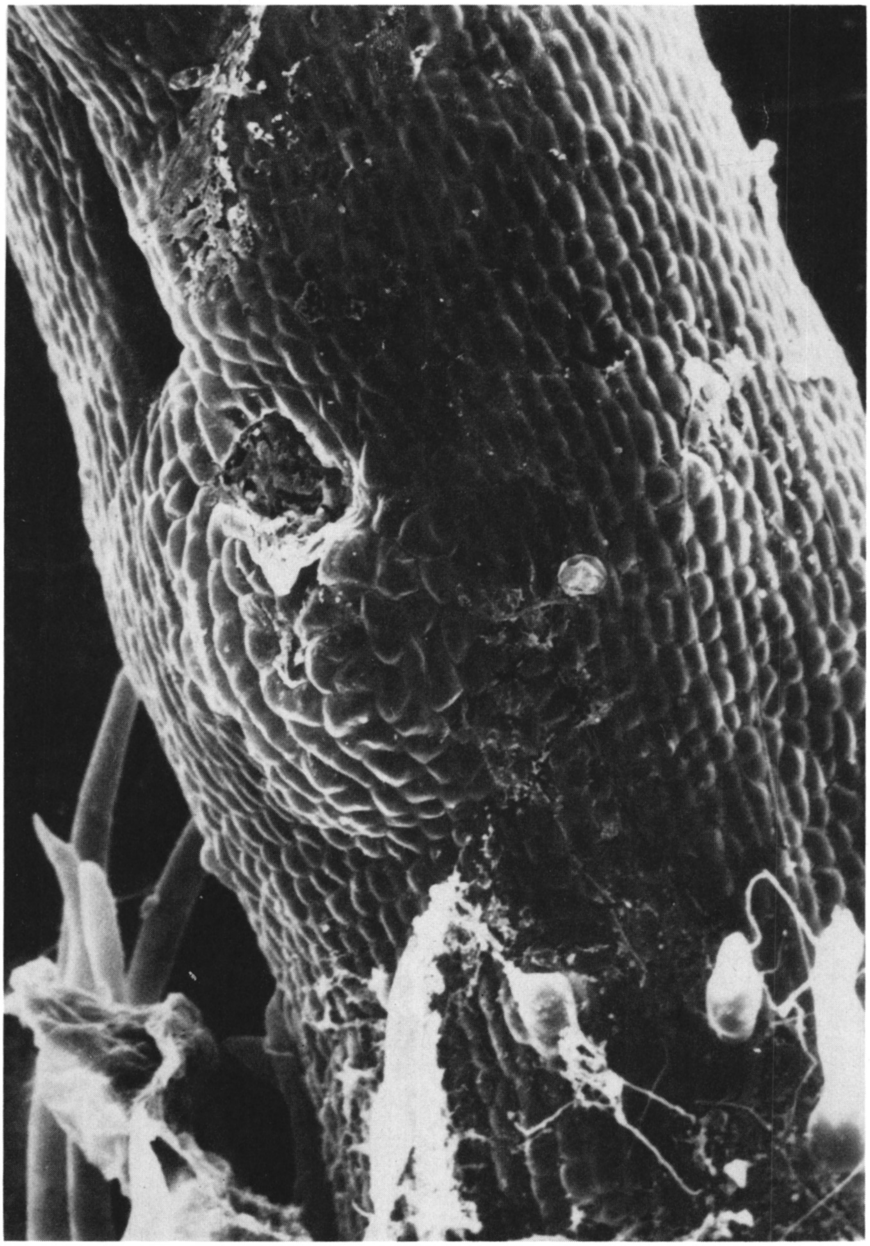

FIGURE 3. Scanning electron micrograph of a cotyledonary gland of *Gunnera chilensis* 6 d after germination. Note the small flap of epidermal cells which has peeled away to expose glandular tissue. (Magnification × 250.) (Photograph courtesy of E. Hoffmann.)

particular attention has been paid to the investments of *Nostoc* cells in recently colonized *Gunnera* cells, since the investments should provide some clues to the manner in which the *Gunnera* cells are colonized. It is agreed that the *Nostoc* cells in healthy, recently colonized glands are surrounded by a membrane of *Gunnera* origin, and that the membrane appears similar to the plasma membrane in staining and thickness. Less agreement exists about whether cell wall material of *Gunnera* origin surrounds the *Nostoc* cells. Neumann et al.[4] believed that wall material comes to surround the cells as they penetrate *Gunnera* cells.

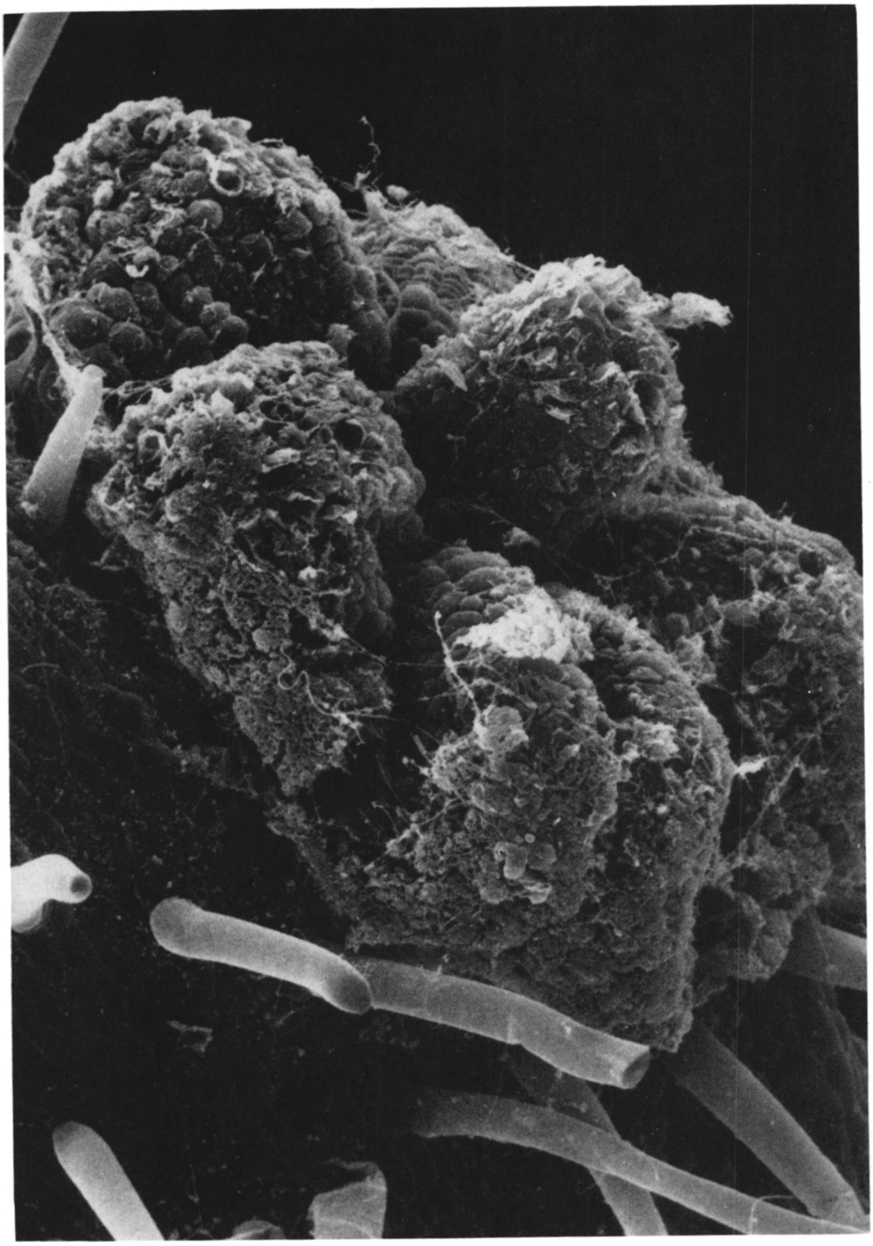

FIGURE 4. Scanning electron micrograph of an older gland, showing a central papilla and surrounding papillae. (Magnification × 135.) (Photograph courtesy of E. Hoffmann.)

Silvester and McNamara[1] reported that wall material was present only around degenerating cells and therefore was produced by the *Gunnera* cell well after colonization was completed. They viewed the presence of wall material as an indicator of senescence. Towata,[12] in a morphometric analysis of glands of different ages, found wall material of *Gunnera* origin surrounding many of the *Nostoc* cells in all the glands examined. She also found as high a proportion of degenerated cells in young glands as in other glands. The presence of *Gunnera* cell wall material around *Nostoc* cells could therefore be explained by the walling off of senescent *Nostoc* cells, as suggested by Silvester and McNamara.[1] The differing data, micrographs, and interpretations of these workers could be reconciled by noting that Silvester and McNamara worked on a small, stoloniferous species; Neumann et al. on a large, rhizomatous species; and Towata on an upright, herbaceous species. However, the uniformity of glandular structural features which has been reported in studies of such variety of species in this genus leads to the expectation that more uniformity should be displayed in the behavior of *Nostoc* cells in the intracellular environment of *Gunnera*.

While *Nostoc* is generally accepted as an endosymbiont, the possibility remains that the cyanobacteria lay within membrane-delimited fingers extending into the *Gunnera* cell interior. Neumann et al.[4] reported seeing continuities between the peripheral cell wall of the *Gunnera* cell and the cell wall surrounding *Nostoc* cells that were residing in an apparent intracellular position. While serial sections would be needed to come to a definitive conclusion, the absence of such continuities in materials studied by Silvester and McNamara[1] and by Towata[3] was taken as further evidence supporting an intracellular location for *Nostoc*.

The necessity to transit the cell wall has led to the suggestion that microorganisms other than *Nostoc* may be essential for colonization by production of cell-wall-degrading cellulases or pectinases,[3,9] since cyanobacteria are not known to produce these enzymes.[13] Clearly, this interesting suggestion could be investigated through the use of *in vitro* culture conditions with axenic *Nostoc* cultures and sterile *Gunnera* seedlings. Neumann et al.[4] reported that they obtained colonized seedlings under such conditions, but both the *Gunnera* seedlings and the *Nostoc* cells were grown on media without organic addenda. *Gunnera* plants, maintained in culture for 7 months, were inoculated with 30-d-old *Nostoc* cell suspensions isolated 4 months earlier. No data were presented to support the assertion that their isolates were axenic over such durations in culture. Considering the difficulties associated with isolating bacteria-free *Nostoc* cells and with sterilizing *Gunnera* seeds, the experiment of Neumann et al. bears repeating because of its significance to hypotheses concerning the mode of entry of *Nostoc*. However the issue of cooperativity between microorganisms is resolved, the colonization process culminates in *Gunnera* glandular tissue which is axenic except for *Nostoc* cells.

Finally, the role of the mucus must be delimited before the events leading to endosymbiosis can be understood. Most investigators studying the symbiosis have recognized that it must play an important role. Several have speculated on the role, but no investigations have been described which test the essentiality and function of mucus in the symbiosis, nor has its chemical constitution been reported. Among the possibilities for its role are one or more of the following: (1) protection of the exposed glandular tissue once the epidermis is no longer present, (2) prevention of desiccation of the population of *Nostoc* growing epiphytically on the shoot apex, (3) nutrition of *Nostoc* cells living epiphytically on the *Gunnera* apex, (4) inhibition of growth of microbes which might otherwise cause disease and decay of the exposed *Gunnera* tissue, (5) provision of a viscous medium through which the *Nostoc* cells might migrate on their way through the channel junctures towards interior regions of the gland, and (6) prevention of differentiation of *Nostoc* cells into heterocysts, which might fragment the filaments or otherwise impair their ability to penetrate the *Gunnera* cell wall.

FORMATION OF A NITROGEN-FIXING TISSUE FROM COLONIZED *GUNNERA* CELLS

A functional, nitrogen-fixing gland in at least some of the large, rhizomatous species of *Gunnera* consists of a sizeable mass of colonized cells extending several millimeters across in every direction. The increase in size of colonized tissue may occur by division of colonized cells or by ongoing colonization of newly produced cells at each cluster. While there is no evidence to help decide between these alternatives, the latter alternative should enable the process of cell penetration to be more easily visualized than appears to be the

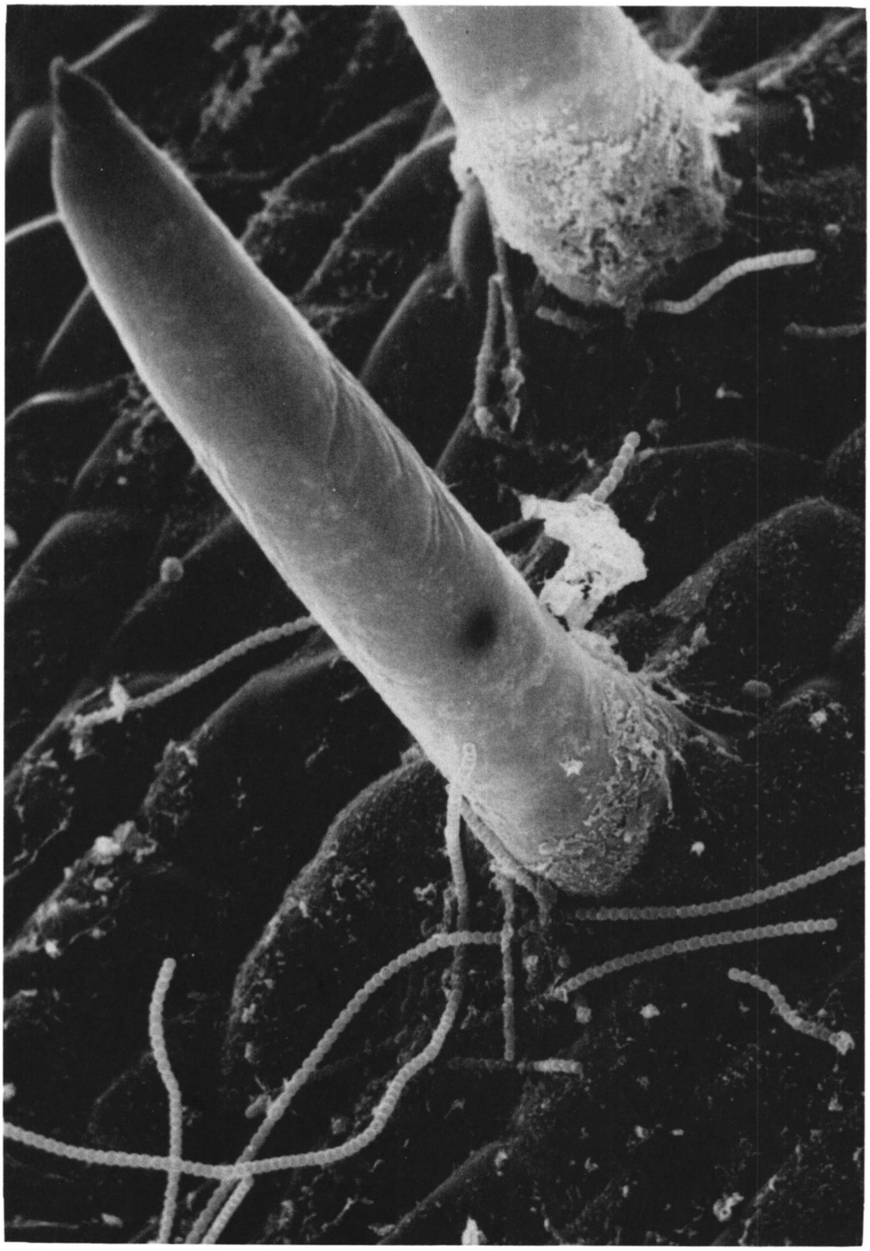

FIGURE 5. Scanning electron micrograph of *Gunnera chilensis* epidermis with hairs in region between apex and cotyledonary glands. Note filaments of *Nostoc* on the surface of the epidermal cells. (Magnification × 700.)

case, since it would occur for every colonized cell. The former alternative would predict that developing glands should contain colonized cells which are undergoing cytokinesis, which has not been described. However, Silvester and McNamara[1] did state that the proposed site of colonization at the base of the channel junctures was occupied by meristematic cells. They attributed the separate clusters of *Nostoc*-containing cells to the rapid growth of colonized cells.

Nostoc cells on the surface of the *Gunnera* apex (Figure 5) as well as within the channel junctions of glands (Figure 6) exist as members of motile filaments. *Nostoc* cells within

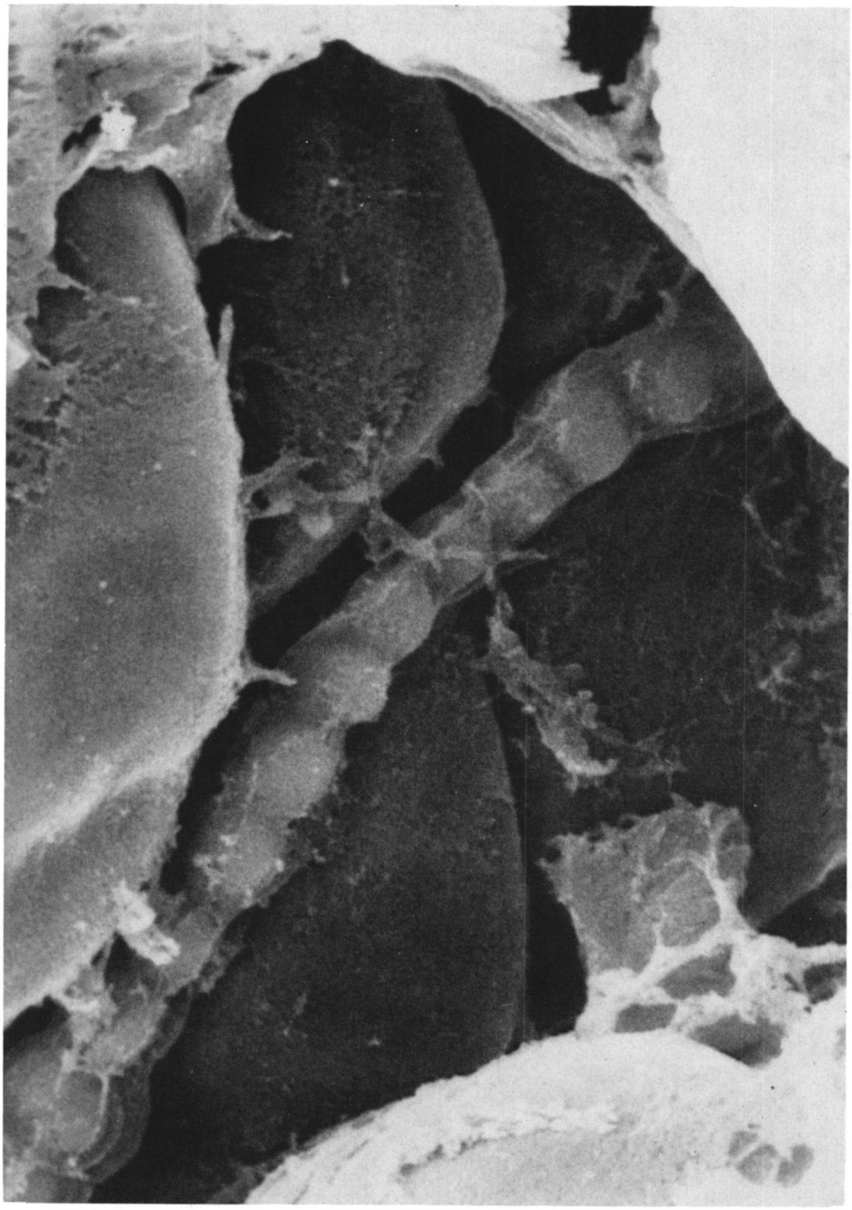

FIGURE 6. Scanning electron micrograph of *Gunnera* cells within a channel juncture of a gland, 48 h after inoculation of a seedling with *Nostoc*. Note *Nostoc* filament and sheathing material surrounding the filament. (Magnification × 3100.)

recently colonized cells also appear as filaments.[11] Thus, entire filaments are presumed to enter *Gunnera* cells. Schaede[9] reported that intracellular filaments became constrained by the confines of the *Gunnera* cell and flexed as a consequence of cell multiplication. In contrast, Silvester[14] reported that the population of *Nostoc* cells became more aseriate as they multiplied in the intracellular environment. He also found that the proportion of heterocysts among the total intracellular *Nostoc* population increased with age of the gland, reaching a proportion of around 65% heterocysts. Towata,[12] on the other hand, reported that the frequency of heterocysts did not change over the span of the plant axis which she investigated.

DEATH

One may speculate that the assumption of an intracellular existence is not without its cost to *Nostoc* and that adjustment eludes many colonizers, which then die.[12] Dead *Nostoc* cells within otherwise healthy-appearing *Gunnera* cells have been reported by all who have studied glands by electron microscopy. Dead *Nostoc* cells often lie next to others which appear perfectly healthy.[4] In the extreme, Schaede[9] described the case in which all *Nostoc* cells were dead in a *Gunnera* cell which still appeared healthy. Of the more recent studies, Silvester and McNamara[1] did not find many dead *Nostoc* cells within glands until they became senescent. On the other hand, Towata[12] found a significant proportion of dead *Nostoc* cells in all the glands she examined, including recently colonized ones.

In addition to death of colonized cells, the longevity of the gland as a functional whole needs to be addressed. Schaede[9] described the existence of healthy-appearing pockets of *Gunnera* cells in portions of the rhizome of *G. macrophylla* that were several years old. Silvester,[14] however, reported that glands ceased to show nitrogenase activity prior to obvious changes in the structure of *Nostoc* or *Gunnera* cells. Thus, assessment of functional capability should accompany evaluations based on structural features. Clearly, the fate of glands which are not colonized, the consequences of *Nostoc* cell death throughout the functional life of the gland, and the cessation of function of the gland are topics that have received insufficient investigation.

SPECIFICITY OF THE SYMBIOTIC ASSOCIATION

Although both *Nostoc* and *Gunnera* are capable of thriving independently of each other, the fact that they form a symbiotic association in which *Nostoc* is intracellular suggests that the two organisms have adapted uniquely to each other. The *Gunnera* cell tolerates a living, multiplying endophyte and the *Nostoc* cells adjust to being screened from the outside world by *Gunnera* membranes. Both organisms might be expected to possess recognition mechanisms to promote establishment of the symbiosis. To further understand the factors which define the taxonomic limits and competence of cyanobacterial isolates to form symbioses with *Gunnera*, a range of *Nostoc* and closely related *Anabaena* species was investigated.[15] The range of cyanobacteria acceptable to *G. manicata* was found to be circumscribed by the genus, *Nostoc*, but included six different isolates within that genus, some from other symbiotic associations and some free-living isolates. Neumann et al.[4] tested two other *Nostoc* species as well as *Anabaena* and failed to get colonization of *G. chilensis*. In experiments by Bonnett and Silvester, support was obtained for the existence of differences in effectiveness between *Nostoc* isolates founded on their variable success in the extracellular and intracellular aspects of the symbiosis.[15] Similar reconstitution studies using a variety of species of *Gunnera* have not been reported.

PHYSIOLOGY OF THE SYMBIOTIC ASSOCIATION

Gunnera plants colonized by *Nostoc* are able to reduce sufficient nitrogen for their own needs.[16] Estimates of the contribution of nitrogen from *Gunnera* to habitats in which it occurs range from field measurements of 12 to 21 kg N per hectare per year for *G. macrophylla*[2] to laboratory-derived estimates of up to 72 kg N per hectare per year for *G. dentata*.[16] Rates of nitrogen fixation are very high due to the high proportion of heterocysts in the gland tissue.

In the large-leaved rhizomatous species, glands function under several layers of filters, starting with the rhizome tissue external to the colonized cells, continuing with the persistent leaf bases from senesced leaves, and ending with the full canopy of thick, broad leaves.

Many researchers have assumed, therefore, that the light levels reaching *Nostoc* cells are much too low to support autotrophic growth. In fact, *Nostoc* cells isolated from *G. macrophylla* have been shown to grow heterotrophically.[17] Furthermore, Silvester[14] has shown that *Nostoc* cells within the gland fix very little CO_2, do not have any detectable phycocyanin, and appear to lack photosystem II activity. Nonetheless, light promoted the rate of acetylene reduction in slices of colonized glands, presumably by enhancing the levels of ATP formation in heterocysts through photosystem I activity.

Since *Gunnera* plants can grow without fixed nitrogen in the soil, the nitrogen fixed by *Nostoc* must be readily assimilated into the plant. Silvester and Smith[16] have shown that ^{15}N fixed by *Nostoc* can be detected in alcohol-insoluble extracts of *Gunnera* tissues, indicating that fixed nitrogen has been transferred and assimilated into *Gunnera* proteins. With the exception of the studies of Silvester and his students, biochemical factors and regulatory mechanisms which promote heterocyst differentiation and transfer of fixed nitrogen from heterocyst to *Gunnera* tissue have barely been investigated in this symbiotic association.

SIGNIFICANCE AND PROSPECTS FOR THE FUTURE

All major food crops are angiosperms. Intensive efforts are underway to understand the legume-*Rhizobium* symbiosis as well as symbioses involving Actinomycete nodule production and free-living bacterial dwelling in the rhizosphere of tropical grasses. *Gunnera* has not been well studied, perhaps because of its obscurity. However, the *Nostoc-Gunnera* symbiosis merits thorough investigation because (1) the host is an angiosperm; (2) *Nostoc* occurs as an endosymbiont in *Gunnera*; (3) *Nostoc* heterocysts, which differentiate within *Gunnera* cells, are able to synthesize or assemble all the factors necessary for nitrogen fixation and to regulate their own internal oxygen tension; (4) *Nostoc* enables *Gunnera* to grow as a luxuriant herb in soils deficient in fixed nitrogen; and (5) the rhizomatous growth habit of many species of *Gunnera* is not unlike that of numerous crop plants.

Many crucial elements of this symbiosis remain unresolved and yet may be productively researched with current techniques and equipment. Fortunately, interest in this symbiosis is growing. In a few years, some of the uncertainties discussed in this review should be resolved, such as the manner in which *Nostoc* cells establish themselves as endosymbionts, the nature and function of mucus produced by *Gunnera*, and the biochemistry of nitrogen transfer and assimilation. Answers to these questions are desirable in the interest of understanding this unique symbiosis and of assessing all possible approaches to agricultural improvement with respect to nitrogen supply and utilization.

REFERENCES

1. **Silvester, W. B. and McNamara, P. J.,** The infection process and ultrastructure of the *Gunnera-Nostoc* symbiosis, *New Phytol.*, 77, 135, 1976.
2. **Becking, J. H.,** Nitrogen fixation in some natural ecosystems in Indonesia, in *Symbiotic Nitrogen Fixation in Plants,* Nutman, P. S., Ed., Cambridge University Press, Cambridge, 1976, 539.
3. **Towata, E. M.,** Mucilage glands and cyanobacterial colonization in *Gunnera kaalensis* (Haloragaceae), *Bot. Gaz.*, 146, 56, 1985.
4. **Von Neumann, D., Ackerman, M., and Jacob, F.,** Zur Feinstruktur der endophytischen Cyanophyceen von *Gunnera chilensis.* Lam., *Biochem. Physiol. Pflanz.*, 161, 483, 1970.
5. **Gusev, M. V., Korzhenevskaya, T. G., Pyvovarova, L. V., Baulina, O. I., and Butenko, R. G.,** Introduction of a nitrogen-fixing cyanobacterium into tobacco shoot regenerates, *Planta,* 167, 1, 1986.
6. **Becking, J. H.,** Dinitrogen-fixing associations in higher plants other than legumes, in *A Treatise on Dinitrogen Fixation Section. III. Biology,* Hardy, R. W. F. and Silver, W. S., Eds., John Wiley & Sons, New York, 1977, 185.

7. **Silvester, W. B.**, Dinitrogen fixation by plant associations excluding legumes, in *A Treatise on Dinitrogen Fixation Section. IV. Agronomy and Ecology,* Hardy, R. W. F. and Gibson, A. H., Eds., John Wiley & Sons, New York, 1977, 141.
8. **Miehe, Von. H.**, Entwicklungsgeschichtliche Untersuchung der Algensymbiose bei *Gunnera macrophylla* Bl., *Flora,* 117, 1, 1924.
9. **Schaede, V. R.**, Über die Blaualgensymbiose von *Gunnera, Planta,* 39, 154, 1951.
10. **Hoffmann, E.**, The *Gunnera-Nostoc* Symbiosis: A Developmental Study of the Uninfected Gland, Master's thesis, University of Oregon, Eugene, 1985.
11. **Sprent, J. I.**, *The Biology of Nitrogen-Fixing Organisms,* McGraw-Hill, London, 1979, 42.
12. **Towata, E. M.**, Morphometric and cytochemical ultrastructural analyses of the *Gunnera kaalensis/Nostoc* symbiosis, *Bot. Gaz.,* 146, 293, 1985.
13. **Fogg, G. E., Stewart, W. D. P., Fay, P., and Walsby, A. E.**, *The Blue-Green Algae,* Academic Press, London, 1973.
14. **Silvester, W. B.**, Endophyte adaptation in *Gunnera-Nostoc* symbiosis, in *Symbiotic Nitrogen Fixation in Plants,* Nutman, P. S., Ed., Cambridge University Press, Cambridge, 1976, 521.
15. **Bonnett, H. T. and Silvester, W. B.**, Specificity in the *Gunnera-Nostoc* endosymbiosis, *New Phytol.,* 89, 121, 1981.
16. **Silvester, W. B. and Smith, D. R.**, Nitrogen fixation by *Gunnera-Nostoc* symbiosis, *Nature (London),* 224, 1231, 1969.
17. **Harder, R.**, Ernährungsphysiologische Untersuchungen an Cyanophyceen, hauptsächlich dem endophytischen *Nostoc punctiforme. Z. Bot.,* 9, 145, 1917.

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Chapter 8

ARTIFICIAL ASSOCIATIONS

Michael V. Gusev and Tamara G. Korzhenevskaya

INTRODUCTION

At present, investigations into the symbioses of cyanobacteria have entered a new stage. This includes dissociation and reconstitution experiments on natural symbioses involving higher plants and symbiotic and free-living cyanobacteria.[1-4] These investigations are of great importance for the understanding of such problems as symbiont specificity and evolution, and the possibility of creating new and novel symbioses with desirable characters.

This new trend of studies on natural symbioses is intimately related to the establishment of artificial associations. To obtain artificial associations, use is primarily made of plants having no natural symbionts. Cyanobacteria are introduced into populations of cultured cells or into isolated protoplasts of higher plants. These studies have been partly discussed in recent reviews.[5-11] Most of the listed papers deal solely with the result of introducing cells or organelles of microorganisms, including cyanobacteria, into isolated protoplasts to obtain endosymbiotic (intracellular) associations. However, these publications have not provided a detailed picture of experiments carried out in the past few years for producing artificial associations of cyanobacteria with plant (tissue) cell cultures and plant regenerates.

This chapter presents a review of studies on the establishment of artificial associations with isolated protoplasts as well as with cultures of higher plant cells and tissues. The studies on producing artificial associations with cyanobacteria were carried out in parallel with similar investigations using other microorganisms. The objectives set in all these studies have much in common. Moreover, some experiments with other microorganisms have formed the basis for successful tests with cyanobacteria. Therefore, the results of work on establishing artificial associations with cyanobacteria will be considered along with experiments involving various other groups of microorganisms.

ARTIFICIAL ASSOCIATIONS AS A MEANS OF MODIFYING CELLS AND PLANTS

The establishment of artificial associations appears to be the creation of new cells and cell systems by introducing microorganisms into a cell, or into populations of cultured cells, of higher plants. Experimentally produced cell systems are called associations by analogy with the associations formed in nature between organisms of different species.[12] At the same time, researchers aim at controlled establishment of intracellular (endosymbiotic) or intercellular (exosymbiotic) associations. In the first case, microorganisms are introduced into isolated protoplasts of higher plants. In the second, attempts are made at joint culturing of plant cells or tissues with microorganisms. However, as will be subsequently seen, the location of microorganisms which is initially predetermined in the experiment — inside cells or in intercellular spaces — cannot always be maintained during establishment or culturing of such systems.

The establishment of associations based on isolated protoplasts or cultured cells of higher plants, on the one hand, and microorganisms, on the other, is regarded at present as one of the new methods of modifying plant cells or whole plants. It is believed that plant cells or cell populations should acquire new properties due to the presence of microorganism cells in them. If an isolated protoplast after uptake of microorganisms will maintain the possibility to regenerate a cell, it creates a prerequisite for cell modification. Culturing of plant cells together with microorganisms would make it possible to produce populations of plants cells with new properties acquired as a result of interaction with microorganism cells. And, finally, the ability of a plant cell *in vitro* to recreate the whole plant opens up a possibility of a controlled plant modification. Obviously the latter is possible, provided that the microorganisms introduced into the cells or cell populations will be preserved in the plants regenerated from such cells.

Artificial associations of higher plant cells could be used for solving the fundamental research problems and practical tasks presented below.

EXPERIMENTAL VERIFICATION OF ENDOSYMBIOTIC THEORY OF EUKARYOTIC CELL ORIGIN

According to current concepts, all eukaryotes have formed as a result of symbiosis between distant species of prokaryotes. Photosynthetic eukaryotes have evolved via the formation of associations between photosynthetic prokaryotes (protoplastids) and heterotrophic microorganisms. Cyanobacteria, like the current *Gloeocapsa* and *Synechocystis*, are believed to have been involved in the formation of red algae and protozoan (*Glaucocystis* and *Cyanophora*) plastids.[12] Moreover, the photosynthetic partners might have been acquired as exo- and then as endosymbionts.[13] Experimental studies on different formation stages of artificial cell systems and the establishment of stable associations would make it possible to employ this approach to investigate the evolution of cells and mechanisms underlying the acquisition of their new properties as a result of symbiosis.

RECONSTITUTION OF CERTAIN EVOLUTIONARY STAGES IN SYMBIOGENESIS

The currently existing symbioses of higher plants with microorganisms have originated as a result of a long and complex evolutionary process. There are data suggesting that some natural associations are currently in the process of formation. The formation of symbioses under laboratory conditions is an extremely rare phenomenon; this is accounted for either by lack of studies in this direction or by the unique nature of this phenomenon per se.[12] Reconstitution of natural symbioses under laboratory conditions or establishment of artificial associations would make it possible to gain an insight into some problems, including the causes underlying the susceptibility of some groups of organisms to symbiosis, the mechanisms of evolutionary origin of symbioses, and conditions for their formation in nature anew. An "enigmatic" property of a small number of eukaryotic species is their ability to form symbiotic associations only with cyanobacteria of the genera *Nostoc* and *Anabaena*.[14-16]

MODELING AND STUDIES OF NATURAL SYMBIOTIC RELATIONS AT CELLULAR LEVEL

Symbioses are of great applied significance in supplying the plants with combined nitrogen in natural ecosystems and agrophytocenoses. Experimental studies on the physiology of symbiotic nitrogen fixation in a whole plant are impeded by the complexity of natural symbiotic systems. An alternative to using whole plants is to study symbiosis with microorganisms in organ, tissue, and cell cultures where the experimental conditions can be subjected to a rigid control.[17]

Significant advances in the studies of symbiotic nitrogen fixation in legumes have been made in the establishment of artificial associations of rhizobia with cell cultures of leguminous and nonleguminous plants. The use of model systems has made it possible to understand the process of nitrogen fixation in rhizobia and the functioning of the natural symbiosis in legumes. Studies on artificial associations have revealed that the genes needed for the fixation of molecular nitrogen are located in rhizobial cells and their expression requires plant metabolites.[6,18]

The use of associations between the cultured cells of higher plants and nitrogen-fixing cyanobacteria would probably make it possible to understand the causes underlying the specificity of cyanobacteria in natural symbioses. The entry of a small number of higher plant species into symbiosis with cyanobacteria may spring from the ability of the former to produce specific factor(s) which may in some way lead to an inhibition of glutamine synthetase in cyanobacteria.[14] This, however, has not yet been proven.[16] The use of experimentally produced cell associations would facilitate the verification of this hypothesis by experiments with artificial inhibition of glutamine synthetase, as well as by a search for cell cultures inducing a similar effect in cyanobacteria.

Two other possibilities of using artificial associations with microorganisms will be considered below. They are most frequently discussed in studies on the establishment of artificial associations based on plant cell cultures and are related to biotechnology.

INCREASE IN THE PRODUCTIVITY OF PLANT CELL CULTURES

Plant cells in culture retain their ability for the biosynthesis of secondary products characteristic of the whole plant. These include such valuable substances important for medicine and industry as polyphenols, alkaloids, glycosides, steroids, terpenoids, ester oils, and many others.[19-21] A shift from scientific developments to commercial manufacture of products using plant cell cultures is currently taking place.[21-24]

One of the requirements for the profitability of production based on plant cells is the possibility of culturing on nutrient medium simple in composition. At the same time, plant cell cultures require the presence of vitamins, phytohormones, amino acids, sucrose, and other substances in the medium.[25]

A considerable restriction in the practical use of plant cell cultures appears to be the energy supply for biosynthetic processes. The plant cells in culture grow heterotrophically and have a limited ability for photosynthesis. It hardly seems possible to combine in a cultured cell the ability for its own photosynthesis and biosynthesis of secondary products.[26] One possible solution to this problem might be the introduction into plant cell cultures of microorganisms capable of biosynthesizing substrates for their growth or precursors for biosynthesis. Introduction of phototrophic microorganisms into heterotrophically growing cells or cell populations seems to play a special part in this. The indicated microorganisms might supply the plant cells with products of photosynthetic carbon fixation. This would allow simplification of medium composition.

The principles underlying the organization of natural biological systems, especially those based on interrelationships of organisms of different species, have not found a sufficiently wide use in biotechnology. Only microbiology has gained a positive experience in coculture. Studies on cocultures of microorganisms (isolated from nature or experimentally produced) have revealed that it is possible to accomplish more effectively (as compared to monocultures) the accumulation of biomass, the cooperative biosynthesis of final products, or the transformation of the original substrate in the desired direction. Such systems find an increasingly wide use in the microbiological industry and can be employed for waste water treatment and biosynthesis of proteins (enzymes) and biologically active substances such as auxins, vitamins, antibiotics, etc.[27] It is believed that biotechnology will use mixed populations diverse in their composition, beginning with combinations of several strains to the combinations of representatives of different kingdoms, i.e., animal and plant kingdoms.[28]

IMPROVEMENT OF AGRICULTURAL PLANTS

Another possibility of using in biotechnology the associations of higher plant cells with microorganisms, which is widely discussed in the literature, consists of producing plants capable of fixing molecular nitrogen.

Increase in the yield of agricultural plants depends in a large measure on supply of combined nitrogen. The plants obtain this in two ways. One is the uptake of chemical nitrogenous fertilizers introduced into soil. Industrial production of nitrogenous fertilizers requires large energy inputs. Their production by a chemical method requires up to 1.6% of world energy, using mostly oil and gas, the reserves of which are diminishing. The plants only partly utilize nitrogenous fertilizers, i.e., up to 50% of the amount applied.[29] Nitrogenous fertilizer transformation products in soil form toxic compounds contaminating the environment and affecting the health of man.[30,31]

Another source of nitrogen for plants is the biological fixation of molecular nitrogen by many groups of microorganisms. The biological fixation of molecular nitrogen is entirely harmless for man and environment. The greater part of nitrogen in nature is fixed by symbiotic nitrogen fixers.[32] They utilize macrosymbiont photosynthesis products to make up for energy input for nitrogen fixation and transfer the combined nitrogen to plants. However, only definite species of plants and microorganisms have acquired an ability to form nitrogen-fixing symbioses in the evolutionary process. Symbioses which account for the greatest part of nitrogen fixation on cultivated soil include those of legumes with rhizobia. Among plant-cyanobacterial symbioses, only that of the water fern *Azolla* with *Anabaena azollae* has significant potential as a nitrogen source in agriculture[33] (see also Chapter 5).

In the last few years, this has aroused the interest of researchers studying the problem of increasing the share of biological nitrogen in plant nutrition. The practice of agricultural production has posed the problem of imparting the ability of nitrogen fixation to economically important plant species which lack this ability. Various approaches have been put forward for the solution of this problem. The following briefly considers some of these approaches and brings out the advantages resultant from the solution of the given problem by cell engineering methods.

In recent years there has been a rising interest in increasing fixed nitrogen by artificial inoculation of plants with nitrogen-fixing bacteria.[34] To obtain more efficient nitrogen-fixing associations with higher plants, microorganisms are introduced into the soil and rhizosphere. However, the resultant effect of a higher yield of plants inoculated with bacteria turns out to stem not only from nitrogen fixation by the bacteria. Yield responses to inoculation are also due to increase in the number of roots under the action of the bacteria and more active uptake of ions and water by the plants. This approach seems to be unpromising due to low survival and ousting of the introduced pure cultures of microorganisms by soil microflora.

Another approach to the solution of the problem under consideration is to use genetic engineering methods for the creation of plants capable of nitrogen fixation. In this case it is suggested that nitrogen-fixing genes (*nif* genes) should be transferred from nitrogen-fixing microorganisms directly into cereals or any other economically important plant species.

The possibility of transferring the *nif* genes into plant protoplasts by means of certain vectors has been discussed.[6,35,36] Subsequent culturing of protoplasts and their regeneration into whole plants would make it possible to produce plants which would carry the introduced genetic information in their cells and possibly in their subsequent generations due to its transfer via seeds.[6] Despite the theoretical possibility of using this approach, however, the employment of genetic engineering will hardly make it possible to cope with the task of producing nitrogen-fixing plants in the near future.[6,35-37] The basic limitations are as follows: it is necessary to develop methods of introducing *nif* genes into a plant cell and of their replication and expression there; higher plants lack the systems which would supply energy for the nitrogen-fixing enzyme nitrogenase (the nitrogen-fixation process requires a large amount of cell energy); the plant cell has no appropriate systems of transport and storage for iron and molybdenum ions at a high concentration which are necessary for nitrogenase synthesis; and the plant cell has no system for protecting nitrogenase from inactivation by oxygen.

The limitations of the two indicated approaches to imparting the ability for fixing molecular nitrogen to plants can be overcome at least partly by introducing the whole cells of nitrogen-fixing microorganisms into a plant. The point is an alternative way of solving the problem under consideration, which is related to the construction of new artificial associations. Such systems should be based on the symbiotic relationships of plant and microbial partners. These experiments should take into account the specific features in the organization of natural nitrogen-fixing symbioses, particularly (1) preservation of the intactness of both partners; (2) integration of the partners within a macrosymbiont organism; and (3) relative isolation of the microsymbiont, its compartmentation in special structures (for instance, location of rhizobia in the nodules of legumes, and cyanobacterium *Anabaena azollae* in leaf cavities of fern *Azolla*). Nitrogen fixation in such experimentally produced systems would take place due to functioning of nitrogen-fixing microorganisms in plant cells or tissues.

According to many researchers, this can be achieved by using a cell engineering technique. Introduction of nitrogen-fixing bacteria into cells or cultured cell populations of nonleguminous plants makes it possible to test a large number of partner combinations and to select stable associations at the level of cultured cells. In the process of coculturing, the partners can adapt to their coexistence. This adaptation may be similar to the changes which the components of natural symbiotic associations undergo as compared to free-living forms. The "usefulness" of such associations can apparently be evaluated only after a successful regeneration of plants from mixed cultures.[38] Plant regeneration by the induction of organogenesis, provided that cell interactions of the partners are preserved, would enable one to cope up with the task under consideration, i.e., the incorporation of a bacterial symbiont into plant tissues (cells) and their integration into the host cell metabolism, with the introduced symbiont preserved intact.

As noted in one of the first studies in this direction, when establishing intracellular-type associations with the use of isolated protoplasts and cyanobacteria,[39] experiments should solve the following questions:

1. It is possible to introduce nitrogen-fixing bacteria into a plant cell?
2. What is the fate of both partners after the uptake of a bacterial cell by a plant cell?
3. Will the nitrogen-fixing cell supply nitrogen for the plant cell?
4. It is possible to regenerate a stable nitrogen-fixing plant from a cell?

The latter two questions are faced by experimentalists establishing artificial associations by introducing nitrogen-fixing bacteria not only into the cell, but also into the populations of cultured cells in order to obtain intercellular interactions between plant and microbial partners. It is believed that the plant produced by the induction of organogenesis will hardly contain a symbiont in its every cell and its association with nitrogen fixers will take place only in individual zones or organs of the plant.[40] This follows from the organization of natural symbiotic systems where the biomass of the symbiont is relatively small; for instance, the percentage of cyanobacterial biomass in some symbioses with eukaryotes accounts for only 1 to 2% of the total biomass.[41]

The advantages of introducing the intact cells of nitrogen fixers into plant cells and tissues are obvious. In this case, nitrogenase will be protected from oxygen, which is generated by the plant and necessary for its growth, by the mechanisms in bacterial cells. Special consideration should be given to the use in experiments of nitrogen fixers capable of photosynthesis. Such microorganisms will perform photoassimilation of molecular nitrogen with its transfer into organic compounds of cells. These organisms (such as cyanobacteria) could supply nitrogenase with energy generated in the process of its own photosynthesis.[39,42] In using heterotrophic nitrogen fixers, a challenging question arises; that is, whether a plant in association with bacteria can form sufficient amounts of photosynthetic products to meet the energy requirements of symbiont nitrogenase without damage to its own growth and

TABLE 1
The Use of Isolated Protoplasts to Obtain Associations with Microorganisms

Source of protoplasts	Microorganisms	Ref.
Pisum sativum	*Rhizobium leguminosarum*	55
Parthenocissus tricuspidata	Yeast (cells, protoplasts), cyanobacterium *Anacystis nidulans*	56
Zea mays, Nicotiana tabacum	Cyanobacterium *Gloeocapsa*	39, 42
Pennisetum typhoideum, Sorghum bicolor	*Azospirillum brasilense*	40
Barley, tobacco	Cyanelles from *Cyanophora paradoxa*, cyanobacteria *Nostoc macrozamia, Gloecapsa*	57
N. tabacum	Cyanobacterium *Anabaena variabilis*	58
Allium cepa, Daucus carota	Cyanelles from *Glaucocystis nostochinearum*	59—61
A. cepa	Unicellular cyanobacterium	62
D. carota	Green alga *Chlamydomonas reinhardii* (wall-less mutant)	63
D. carota	Green alga *Stigeoclonium* (protoplasts)	64
N. tabacum	Cyanobacterium *Anabaena variabilis* strain (CALU-458) (cells and spheroplasts)	65

TABLE 2
Artificial Associations of Cultured Tissues and Cells of Higher Plants with Microorganisms

Plant tissue (cell) culture	Microorganisms	Ref.
Glycine max	*Rhizobium*	80, 81
Pisum sativum		82
Alfalfa and other legumes (sweet clover, lupin, vetch, clover, etc.)		83
Brassica napus, Bromus inermis, Triticum monococcum	*Rhizobium*	84
Nicotiana tabacum and other nonlegumes (carrot, petunia, purslane, glasswort, etc.)		85, 86
Daucus carota	*Azotobacter venilandii*	38
Saccharum officinarum	*Azospirillum brasilense*	40, 87—90
Medicago allus, P. sativum, D. carota, T. monococcum, N. tabacum, etc.	*Azospirillum*	91, 92
D. carota	*Chlorella*	60, 61, 93, 94
Ruta graveolens	Fungi: *Rhodotorula rubra, Botrytis allii, Cochliobolus lunatis*, and others	95

deterioration of its nutritional value.[36,37,40,43] Many other properties are also under consideration, which should meet the requirements of nitrogen-fixing microorganisms — the probable partners of higher plants in artificial associations.[43]

All the possibilities listed above are apparently of theoretical importance at the present time. They are still to be evaluated in view of their economic efficiency. Moreover, it is necessary to make a scientific prediction of the ecological impact of increasing nitrogen fixation, which could lead to unfavorable shifts in the nitrogen balance in the biosphere.

SPECIFIC PROPERTIES OF CYANOBACTERIA AS COMPONENTS OF ARTIFICIAL ASSOCIATIONS

To establish artificial associations based on isolated protoplasts (Table 1) or cultured cells (tissues; Table 2) of higher plants, microorganisms of various taxonomic groups have been used. A considerable number of experiments have been carried out using cyanobacteria as partners of plant cells in artificial associations (Tables 1 and 3). These organisms possess

TABLE 3
Use of Cyanobacteria for Production of Artificial Associations with Cell and Tissue Cultures and Regenerates of Higher Plants

Plant species 1	Cyanobacteria species 2	Ref. 3
	Suspension Cultures	
Nicotiana tabacum	*Anacystis nidulans*	8, 10, 111—116
Panax ginseng[a]	*Chlorogloeopsis fritschii*	8, 10, 113, 115, 117—128
P. ginseng[b]	*Anabaena variabilis*[c]	123
P. ginseng[b]	*Calothrix elenkinii*	123
P. ginseng[b]	*Nostoc muscorum*	123
Medicago sativa	*A. variabilis*[c]	115, 123, 129—131
M. sativa	*Chlorogloeopsis fritschii*	123
M. sativa	*Calothrix elenkinii*	123
M. sativa	*N. muscorum*	123
Solanum laciniatum	*A. variabilis*[c]	123, 132
S. laciniatum	*Chlorogloeopsis fritschii*	123, 132—135
Papaver somniferum	*A. variabilis*[c]	123
Dioscorea deltoidea	*Anacystis nidulans*	
Daucus carota	*Anabaena flos-aquae*	61, 94
D. carota	*Plectonema boryanum*	61, 94
D. carota	*Gloeothece* sp.	61, 94
N. tabacum from protoplasts	*A. variabilis*[d]	8, 76, 77
N. tabacum from mesophyll tissue	*A. variabilis*[c]	116, 124, 129, 134, 136—140
N. tabacum	*Anabaena* sp.	141, 142
N. tabacum	*Nostoc* sp.	141, 142
M. sativa	*A. variabilis*[c]	115, 116, 130, 143
	Regenerated Plants	
N. tabacum from protoplasts	*A. variabilis*[d]	144
N. tabacum from mesophyll tissue	*A. variabilis*[c]	115, 116, 129, 134, 136—140, 145
M. sativa	*A. variabilis*[c]	130, 143

[a] Auxotrophic.
[b] Prototrophic in phytohormones strains.
[c] Nitrogen-fixing *A. variabilis* strain (ATCC 29413).
[d] Nonnitrogen-fixing *A. variabilis* strain (CALU-458).

a variety of specific properties by which they could be referred to as the most suitable partners of plant cells in artificial associations. Some of these properties are

1. Cyanobacteria are believed to have played a role in the formation of eukaryotic cells in the evolutionary process via symbioses with nonphotosynthesizing organisms.[12]
2. Cyanobacteria, more often than other phototrophs except for green algae, enter into symbiotic relations with other organisms in nature, forming associations of the endo- and exosymbiotic type.[9,44] This offers grounds for their introduction into both isolated protoplasts and populations of cultured cells.
3. Cyanobacteria perform various metabolic functions in symbioses: with autotrophic organisms (algae, plants) they act as a nitrogen-fixing component of symbiotic systems;

heterotrophic organisms (protozoa, fungi), their basic function consists in supplying the partners with photosynthetic products.[9] Hence, cyanobacteria can be used both as nitrogen-fixing and as phototrophic components of artificial associations.

4. Cyanobacteria excrete various compounds into the environment: carbohydrates, peptides, amino acids, organic acids, vitamins, etc.[45-47] This forms the basis for establishing metabolic interactions with plant cells in culture or in a plant.
5. In the process of photosynthesis, cyanobacteria generate oxygen; the cultured plant cells, on the contrary, require oxygen for their respiration. Coculturing of cyanobacteria and plant cells in the light can thus create more favorable conditions for both organisms.[48]
6. The growth rates of cyanobacteria are comparable to those for higher plant cells in batch cultures; the growth cycle for many suspension cultures, until they reach a stationary phase, is 3 to 4 weeks in either case.[49-50] In the formation of natural symbioses much importance is attached to the coincidence in the growth rates of partners; this may ensure coordination in the growth of the symbiont and the host.[12] When establishing artificial associations this fact is taken into account, and slow-growing bacterial strains are specially selected as partners of plant cells.[51]
7. Cyanobacteria have practical use in supplying plants with combined nitrogen. Symbiosis of fern *Azolla* with *A. azollae* is used in agriculture as a source of combined nitrogen in rice fields.[33] Nitrogen-fixing cyanobacteria play an important part in the accumulation of combined nitrogen in soil. They can provide combined nitrogen to plants and cause gains in plant yield in inoculated soils.[34,44]

The successful use of cyanobacteria in artificial associations with plant cells does not seem so obvious, however, if the following facts are taken into account:

1. Only certain species of cyanobacteria (representatives of the genera *Nostoc* and *Anabaena*) occur in natural associations with a limited number of higher plants and these associations show a high degree of specificity.[41]
2. Many species of cyanobacteria produce toxins affecting various groups of organisms.[44,45,47] One of the requirements for using such microorganisms in artificial associations with higher plants is the avoidance of their toxic effect by the host plant.[39,43]
3. Cyanobacteria and plant cells differ considerably in their requirements for environmental factors: optimum pH values are acidic (5.0 to 5.5) in higher plant cells and neutral-alkaline (7 to 10) in cyanobacteria; optimum temperatures for the growth of cyanobacteria is 24 to 27°C), which is lower than that for plant cells (30 to 40°C); the concentration of mineral salts in the medium for growing plant cells is higher than usually used for culturing cyanobacteria; being autotrophs, cyanobacteria do not require organic compounds in the medium, whereas plant cells require sucrose and other organic compounds.[25,44,52] Indeed, the differences in nutritional and growth factors, along with distant phylogenetic relations of partners, appear to be crucial factors in the establishment of natural symbioses.[53] Hence, the listed differences in the factors needed for the growth of plant cells and cyanobacteria can be referred to as the causes facilitating the formation of artificial associations rather than hampering their formation.

INTRODUCTION OF CYANOBACTERIA AND OTHER MICROORGANISMS INTO PROTOPLASTS OF PLANTS

PARTNERS OF EXPERIMENTAL ASSOCIATIONS

To obtain the intracellular type of associations, numerous attempts have been undertaken to introduce cyanobacteria and other microorganisms into isolated protoplasts of various higher plant species (Table 1), which include cereals and other crops having no natural symbionts. The protoplasts are isolated from plant tissues, or cells in suspension cultures, by the enzymic method.[54]

Isolated plant protoplasts have been used to introduce various species of cyanobacteria: *Anacystis nidulans*,[56] *Gloeocapsa*,[39,42,57] *Anabaena variabilis*,[58,65] *Nostoc macrozamia*,[57] and a unicellular cyanobacterium.[62] Cyanelles isolated from the protozoa *Cyanophora paradoxa*[57] and *Glaucocystis nostochinearum*[59,61] have also been used.

The cyanobacteria used differed in their metabolic properties. *A. variabilis*,[58] *N. macrozamia*,[57] and *Gloeocapsa*[39,42,57] are nitrogen fixers. *Anacystis nidulans*[56] and a nonnitrogen-fixing strain of *Anabaena variabilis*[65] are obligate photoautotrophs. Nitrogen-fixing *A. variabilis* is a facultative phototroph and is capable of heterotrophic growth in the dark.[66] This is believed to ensure the growth of the endosymbiont in the dark, such as during germination of seeds.[58] Along with a wild strain, auxotrophic mutants of *A. variabilis* have also been introduced into protoplasts. This technique is employed to establish a possible dependence of the symbiont on the growth and metabolism of the host cell. Cyanelles appear to be endosymbiotic cyanobacteria with a reduced or no cell wall and are adapted to intracellular existence.[59]

In some experiments, cyanobacterial cells are first subjected to sonication to remove the outer sheath (in *Gloeocapsa*[39]) or to fragment the filaments (in *A. variabilis*).[58] *A. variabilis* cells[65] are treated with lysozyme to obtain spheroplasts.[67,68]

Microorganisms of other systematic groups have also been used for introduction into isolated plant protoplasts, e.g., nitrogen-fixing bacteria (*Rhizobium leguminosarum*[55] and *Azospirillum brasilense*[40]), yeast,[56] and green algae.[63,64] Intact cells of microorganisms and protoplasts obtained from them[56,64] have been used. Due to the similarity of problems posed in experiments with cyanobacteria and other microorganisms, as well as the similarity of the results, all these studies have been summarized in chronological order in Table 1.

MECHANISMS OF PENETRATION INTO PROTOPLASTS

Microorganisms are introduced into isolated plant protoplasts by using special inducing factors. Microorganisms penetrate protoplasts as a result of their uptake by the protoplasts or as a result of their fusion with them. In the case of pea protoplasts obtained from leaf mesophyll cells after enzymic removal of the cell wall, *Rhizobium* cells are taken up directly.[55] In most cases, however, polyethylene glycol (PEG), which is known as an agent increasing the fusion frequency of higher plant protoplasts,[69] must be used as an inducer of uptake or fusion.

Uptake of cyanobacteria and other microorganisms, except green algae, seems to involve a type of endocytosis that is via invagination of plasmalemma.[5] PEG induces the attachment of microorganisms to the plasmalemma, invagination of the plasmalemma around the microorganisms, and subsequent enclosure of the open end of the invagination by the fusion of the plasmalemma. This leads to the release of the resultant vesicule, with microorganism, into protoplast cytoplasm. *Rhizobium* cells enter the vesicles, formed by invaginations of the plasmalemma in the protoplast, during plasmolysis in the process of cell wall degradation under the action of enzymes.[55]

Protoplasts and wall-less mutants of green algae have been shown to fuse with carrot protoplasts in a PEG-mediated process; however, endocytosis has not been observed. In this event, the plasmalemmas of plant protoplasts and the algae are integrated,[63] but the algal organelles released into the protoplast cytoplasm are not surrounded by the plasmalemma of the plant protoplast.[63,64]

Due to the problem of preserving the organelles or microorganisms to be introduced into protoplasts intact, both methods have shortcomings. The uptake via endocytosis leads to isolation of these structures, in the vesicles, from protoplast plasmalemma (Figure 1A).

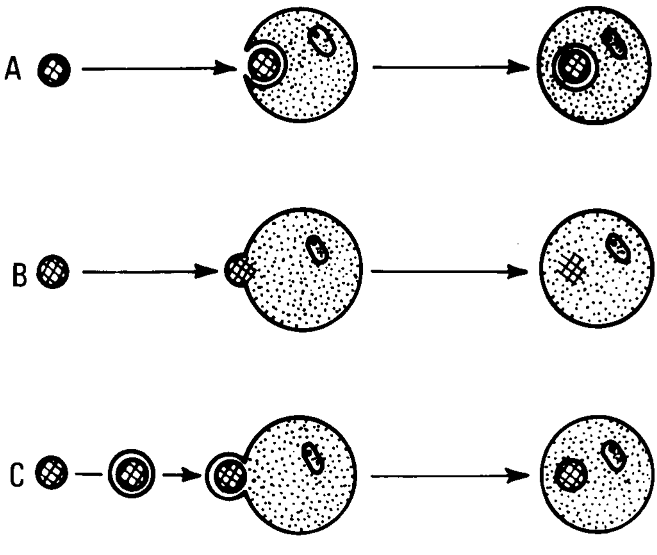

FIGURE 1. Introduction of microorganisms into isolated protoplasts of higher plants. (A) Uptake through membrane invagination (endocytosis). (B) Fusion of membranes. (C) Fusion of protoplast membrane with liposome. (From Fowke, L. C. and Gamborg, O. L., *Int. Rev. Cytol.*, 68, 9, 1980. With permission.)

Because these vesicles may fuse with the lysosomal apparatus of a plant protoplast, there is danger that this will lead to the breakdown of the foreign material introduced.[7] The membrane of an endocytosis vesicle is not preserved in all cases. For example, in the uptake of yeast protoplasts[56] and cyanelles[57] by plant protoplasts, no extra membrane surrounding the microorganism has been observed. This is thought to arise from the fusion of the outer membrane of the microorganism with the membrane of the endocytosis vesicule.[6]

The fusion of a plant protoplast with a microorganism is accompanied by the integration of their membranes, disturbance of the intactness of the microorganism, and release of its organelles into the plant protoplast (Figure 1B). Thus, the fusion of green algae with protoplasts should be regarded as a method for introducing intact organelles rather than whole microorganisms into a plant cell.

An alternative method that avoids the shortcomings of both these methods is enclosure of microorganisms in lipid vesicles/liposomes[5,7] (Figure 1C). This technique has already been used for introduction of unicellular cyanobacteria into onion protoplasts.[62] The advantage of this technique is that the lipid vesicles will fuse with protoplast plasmalemma, thus releasing the intact cells of the microorganisms into protoplast cytoplasm.

EFFICIENCY OF UPTAKE AND FUSION

The number of protoplasts containing microorganisms varies for different systems, ranging from 1 to 20% of the total number of protoplasts used. *Anabaena variabilis* cells are found in 7.6% of tobacco protoplasts[58] and 20% of *Parthenocissus tricuspidatum* protoplasts taken up *Anacystis nidulans* cells.[56] The uptake of cyanelles by barley and tobacco protoplasts is far more frequent than that of cyanobacterial cells and amounts to 20%, whereas for *Nostoc macrozamia* and *Gloeocapsa* this was 2%.[57] Depending on experimental conditions, the uptake frequency may vary greatly and, as shown for the *Gloeocapsa*-maize protoplast system, may be 1[39] or 15%.[42] A small percentage of uptake by protoplasts has been noted for cells and spheroplasts of *Anabaena variabilis*[65] and for cyanobacteria contained in lipid drops.[62] In the latter case, olive oil drops are taken up more frequently than mineral oil drops and PEG treatment enhances the uptake.

The uptake frequency of other microorganisms by protoplasts varies over the same range: 5% in the pea-*Rhizobium* system[55] and 20% in the *P. tricuspidatum*-yeast system.[56]

Removal of the sheath is essential for uptake of cyanobacterium *Gloeocapsa*.[39] It is believed that the absence of cell walls and sheaths may be prerequisites for the interaction of cyanobacteria with the host cells.[57] Indeed, endosymbiotic cyanobacteria (cyanelles) either lack or have greatly reduced cell walls.[44,70]

The number of microorganism cells taken up by one protoplast may also vary. This index is observed to depend on the relationship between the size of protoplasts and that of the cells taken up by them. For instance, electron microscopy of a pea protoplast section shows up to five *Rhizobium* bacteria.[55] Similarly, *P. tricuspidatum* protoplast section shows up to 18 *A. nidulans* cells.[56] In the case of larger *Gloeocapsa* cells, maize and tobacco protoplasts take up no more than one cyanobacterium per protoplast. A higher uptake frequency of the same cyanobacterium is obtained in larger protoplasts.[39]

CHARACTERISTICS OF UPTAKE AND FUSION PRODUCTS

The systems obtained by uptake of microorganisms by protoplasts or by fusion with them has a limited ability for survival or culturing. Tobacco protoplasts containing cells and spheroplasts of *Anabaena variabilis* exhibit considerable destruction of cytoplasm.[65] Maize protoplasts show a rapid movement of cytoplasm 14 h after they take up *Gloeocapsa* cells,[42] which points to their viability.[71] Protoplasts containing *A. variabilis* cells,[58] *G. nostochinearum* cyanelles,[59] or *C. reinhardii* organelles[63] regenerate cell walls during culturing. Only carrot protoplasts, when taking up *C. reinhardii* organelles, are capable of subsequent division.[63] Regenerated tobacco cells with intracellular *A. variabilis* never divide, and in 5 days of culturing they are destroyed.[58] In only one case a tobacco cell containing a cyanobacterium, after 27 d of culturing, has been detected. The uptake of cyanobacterial cells and cyanelles by tobacco protoplasts has been studied in a system capable of regeneration,[57] although no information on the fate of this system has been published so far.

There is evidence for the preservation of the intactness of microorganism cells and organelles inside protoplasts. Pea protoplasts have been found to have dividing *Rhizobium* cells, but it remains to be established whether they start their division before or after their uptake by the protoplasts.[55] The morphology of bacteria points to their intact state. *A. variabilis* cells in the endocytosis vacuoles of tobacco protoplast cytoplasm retain their ultrastructure similar to intact cells, which may attest to the preservation of their viability.[65] No division of another *A. variabilis* strain cells was observed in tobacco protoplasts during their culturing, although the cyanobacterium retained its characteristic blue-green color.[58] None of the auxotrophic mutants of this cyanobacterium had any advantage in the formation of intracellular systems. They died at the same rate as the systems formed with the wild strain. This also attests to the fact that the death of auxotrophic mutants, as a result of their starvation, would appear not to have been the cause of decrease in the number of "hybrid" cells with time. Green alga organelles (nucleus and chloroplasts) in plant protoplasts remain intact for 1 h of observation.[64] However, most of the organelles are degraded during culturing, but the chloroplasts are found in the carrot cell cytoplasm for up to 10 d.[63]

Some authors have expressed regret in connection with lack of systematic studies on the photosynthetic and nitrogen-fixing activity of cyanobacteria introduced into protoplasts, as well as on the metabolic activity of the introduced organelles and microorganisms.[6] A study on the photosynthesis of isolated cyanelles showed that they were unsuitable for endosymbionts in artificial associations. This is due to an almost complete loss of their ability for CO_2 fixation when isolated (isolated cyanelles accounted for only 5% of the activity compared to that in the intact cyanome) and to the inability of experimental cells containing cyanelles for division.[61] An attempt to select nitrogen-fixing associations which would function at the expense of the cyanobacterium *A. variabilis* in tobacco protoplasts proved to be unsuccessful.[58]

Thus, no viable intracellular associations with microorganisms based on isolated protoplasts have been obtained so far. No advantages in the survival of intracellular systems have been found for any of the groups of microorganisms. The failure can be due to the following reasons:

1. The damaging effect of PEG treatment on protoplasts and microorganisms.
2. Adverse effects of special procedures for preparation of microorganism cells for experiments (isolation of protoplasts and spheroplasts from the cells; isolation of cyanelles from host cells; subjecting cyanobacteria to sonication).
3. Biological incompatibility of the selected pairs of partners: either the protoplast is unsuitable as a habitat for a given microorganism or the microorganism exerts a toxic effect with its intracellular presence or secreted metabolites on the plant cell (probable cause of death of tobacco protoplasts containing cells of *A. variabilis*).[58]

Consequently, the successful continuation of studies in this direction requires the search for compatible combinations of partners, selection of an adequate method of introducing microorganisms into protoplasts, developing of "sparing" conditions for inducing uptake (fusion), and improvement of conditions for culturing the products to be obtained.

The dependence of the uptake or fusion of microorganisms with protoplasts on the relationship of their size seems to be one of the factors determining the success in obtaining artificial symbioses. This should be taken into account in selecting partners in such experiments.

Apart from the use of PEG to induce the uptake of microorganisms by protoplasts, a successful application of polyvinyl alcohol (PVA), which is also known as an inducing agent of protoplast fusion,[72] should be noted. PVA-mediated uptake of *Agrobacterium tumefaciens* spheroplasts.[73] *Escherichia coli* cells, and spheroplasts[74] by *Vinca rosea* protoplasts has been shown in experiments carried out for genetic transformation of protoplasts (for this reason the given studies have been omitted in Table 1). In these tests, PVA produced no toxic action on the protoplasts, which was noted in comparative experiments with PEG.

Calcium ions have also been successfully used for the fusion of plant protoplasts.[75] Experiments have shown that the presence of this inducer, as compared to that of PEG, provided a higher frequency of attachment of *Anabaena variabilis* cells (nonnitrogen-fixing strain) to tobacco protoplasts.[76] For instance, while the adhesion of cyanobacteria to protoplasts was a maximum of 35% in the presence of PEG, in the presence of calcium ions, at pH 10.5, it was 90 to 100%.

A higher adhesion of cyanobacteria also enhances the probability of their uptake by protoplasts. Conditions have been selected (0.05 *M* $CaCl_2$, pH 7.5) under which the adhesion is observed in 60% of protoplasts. Both protoplasts and cyanobacteria retain their viability under these conditions, unlike treatment with PEG which produces a strong adverse effect on cyanobacterial protoplasts and spheroplasts.[65] Protoplasts with adsorbed cyanobacteria, after treatment with calcium ions, regenerated cell walls and formed callus in association with the cyanobacterium.[76,77] Since this work involved no selection of protoplasts with intracellular localization of cyanobacteria, the system given has been omitted in Table 1 and will be discussed in more detail later.

There is also a hypothesis that the cells of nitrogen fixers can be introduced into isolated protoplasts by inducing the uptake with an electric field.[78] This method, successfully used for fusing plant protoplasts,[79] has the advantage of exerting no strong adverse effect on the protoplasts as compared to other fusion inducers.

The development of a special technique for introducing organelles and cells of microorganisms into higher plant cells is now underway. For instance, a method of microinjection was used to introduce unicellular cyanobacteria and cyanelles from *G. nostochinearum* into the cavity between the cell wall and the plasmalemma of plasmolyzed onion epidermal cells.[59] This method can be regarded as a stage preceding the entry of cyanobacteria into cytoplasm through induced fusion or uptake, or employed in establishing associations of the intercellular type.

Obtaining mixed cultures of plant cells with microorganisms apparently might be the preliminary stage toward checking the biological compatibility of partners for producing intracellular systems. This is technically more feasible, thus making it possible to check the ability for a joint growth of a great number of combinations of partners.

Prior to considering the achievements in the field of coculturing of higher plant cells and cyanobacteria, basic work carried out with other microorganisms will be addressed. This will enable us to follow the development of coculturing which has formed the basis for successful experiments with cyanobacteria.

ASSOCIATIONS BETWEEN CULTURED CELLS OF HIGHER PLANTS AND MICROORGANISMS

PARTNERS OF ASSOCIATIONS AND THE PRINCIPLES OF THEIR ESTABLISHMENT

To establish associations based on cultured cells and tissues, a diverse group of plants and microorganisms have been used (Table 2). The cell (tissue) cultures were produced from commercially important plants, including producers of natural compounds. Microorganisms are represented by nitrogen-fixing bacteria which include symbiotic (rhizobia) and free-living (*Azotobacter, Azospirillum*) bacteria. To establish associations with callus cultures, green algae *Chlorella* and various fungal species have been used.

The objectives of establishing associations with some microorganisms and the results achieved in these works vary to a great extent. The principle of producing associations which is common for all these studies is joint culturing of plant cells and callus tissues with microorganisms. At the same time, the methods of introducing microorganisms into a system for joint culturing may be different. These include (1) introduction of *Rhizobium, Chlorella, Azotobacter,* and *Azospirillum* cells directly into plant callus or suspension culture (mixed cultures); (2) seeding of fungi on the surface of agar medium adjacent to but not in contact with callus (cocultures); (3) separation of *Rhizobium* and *Azospirillum* cells and suspension or callus culture cells with special filters (membranes) providing exchange of metabolites, but preventing contacts between the cells of the partners (transfilter cultures).

To consider concrete studies in this direction it seems expedient to unite them, depending on the problems, by one or another group of experimental investigations.

ASSOCIATIONS WITH RHIZOBIA

The basic objective in the work on producing associations with rhizobia was first to create simplified model systems to study the symbiotic relationship of a leguminous plant with rhizobia. Due to the diversity of studies on the problem, only those which make up the basic stages in the development of this direction and are of some interest in terms of designing new symbiotic systems will be considered.

Soybean callus tissue was first inoculated with *Rhizobium* cells which caused lignification and differentiation of the plant cells.[80] Later, an association was obtained in which rhizobia formed infection threads and bacteroids in soybean callus culture.[81] In the association with callus cells, the rhizobia manifested acetylene reduction activity (ARA) which they lacked in pure culture. This provided evidence for activation of nitrogenase activity in rhizobia in association with callus cells. (In this and all other studies, unless otherwise specified, nitrogenase activity is determined using ARA.)[96]

Numerous subsequent studies have confirmed the induction of nitrogenase in rhizobia by cell (tissue) cultures of legumes. Essential for understanding the interaction of macro- and microorganisms in natural symbioses of legumes have been the reports of nitrogenase induction in *Rhizobium* by tissue cultures of nonleguminous plants, rapeseed (*Brassica napus*), brome grass (*Bromus inermis*), wheat (*Triticum monococcum*),[84] and tobacco (*Nicotiana tabacum*).[85] Later, this effect was found in associations of *Rhizobium* with tissue and cell cultures of other species (Table 2).

The criterion for establishing associations of rhizobia with cell and tissue cultures of leguminous and nonleguminous plants is the induction of nitrogenase in rhizobia, which is not found in pure cultures, by the plant partner. Moreover, this effect was induced in *Rhizobium* either by the presence of plant cells or the medium conditioned by them. The latter fact was ascertained by culturing *Rhizobium* separately from plant cells. For this purpose they were separated from one another in the process of culturing with special filters (membranes).[85,97,98] Thus, the induction of nitrogenase in *Rhizobium* was found to require factors secreted by cells which are common to both leguminous and nonleguminous plants.

Associations with plant cells and tissues exhibit intensive multiplication of rhizobial cells (without their multiplication in pure cultures on the same media). The intensive growth of *Rhizobium* in this case results not from the presence of living plant cells but from that of their metabolites.[99] Rhizobia are located, as a rule, on the surface of plant cells; they penetrate deep into the callus through intercellular spaces and do not enter intact cells. However, at times they are found in plant cells.[81,100,101] It is believed that the penetration of bacteria into plant cells may result from the induction of cellulase activity in plant cells by *Rhizobium* cells, as found in experiments with soybean cells.[102] This leads to partial degradation of cellulose microfibrils in the plant cell walls, which apparently helps the penetration of bacterial cells into cytoplasm of the plant cells.

Bacterial cells, as a rule, produced an inhibitory effect on the growth and survival of plant cultured cells and tissues. The inhibitory effect of rhizobia is thought to result from the accumulation of hormones produced by the bacteria in superoptimal concentrations.[103] However, there is one reported case where the growth of soybean callus was stimulated.[104] This stimulatory effect is believed to result from the formation of cytokinins by the bacteria rather than from bacterial nitrogen fixation.[104]

There have been sporadic reports of a favorable effect, or the absence of an adverse effect, of rhizobia on plant cells *in vitro*. Using labeled nitrogen, *Portulaca grandiflora* callus tissue was shown to take up nitrogen fixed by rhizobia,[99] which is analogous to the interaction of partners in nodules *in vivo*. There has been only one known successful attempt to regenerate tobacco plants from an association of callus culture with *Rhizobium*.[18] Unfortunately, no information is available on the fate of these plantlets or on their invasion with rhizobia in the process of their regeneration from the callus.

The work on establishing artificial associations in this direction has provided information which is important for designing new cellular systems nonexistent in nature. Establishment of nitrogen-fixing associations via interaction of rhizobia with cells of nonleguminous plants has pointed to the fact that the ability to enter nitrogen-fixing associations is widespread among plants.[105] These experiments have shown that the species barriers in the symbiotic fixation of nitrogen lie at the stage of infection and formation of nodules rather than at the stage of nitrogenase expression.[6,84] The possibility of inducing nitrogen fixation in rhizobia by cultured cells of nonleguminous plants leads to the conclusion that symbiosis with *Rhizobium* may include nonleguminous crops. A conviction has been expressed that various combinations of eukaryotic cells and nitrogen-fixing microorganisms can be used in experiments *in vitro* to overcome the high specificity in natural symbioses and to understand the causes for the selective emergence of natural symbiosis only in some groups of plants.[106]

ASSOCIATIONS WITH NITROGEN-FIXING FREE-LIVING BACTERIA

Experiments with establishing associations of cultured cells (tissues) of leguminous and nonleguminous plants with symbiotic bacteria (rhizobia) have formed the basis for obtaining associations with nonsymbiotic nitrogen fixers *Azotobacter* and *Azospirillum* (Table 2). These heterotrophic soil bacteria are often found in the plant rhizosphere and in some cases form loose associations with them, providing the plants with combined nitrogen.[34,107] In recent years, bacteria of the genus *Azospirillum* have aroused great interest among researchers. In experiments involving artificial inoculation of these bacteria, considerable enhancement of plant growth and nitrogen content has been noted. In establishing artificial associations based on plant tissue cultures with these bacteria, the basic objective has been to produce efficient nitrogen-fixing systems which have the prospect of plant regeneration.

For this purpose, various combinations of plant tissue cultures with *Azotobacter vinelandii* and *Azospirillum brasilense* have been used. The plant cells were found to be species specific in the formation of associations with these bacteria. The callus cultures of *N. tabacum, Pennisetum americanum,* and *Eremochloa ophiuroides* did not survive upon their inoculation with *A. brasilense.*[87] The callus cultures were rapidly "overgrown" with bacteria and died 4 weeks after inoculation. On the contrary, inoculation of sugarcane (*Saccharum officinarum*) tissue with the same bacterium gave rise to an association which was successfully subcultured for 18 months. The stable association was formed only on the medium with no or low content of combined nitrogen.

The bacteria exhibited nitrogenase activity irrespective of whether they formed associations with plant cells and whether the latter survived or died. The cultured plant cells stimulated nitrogenase activity in *Azospirillum* in a similar way to that in *Rhizobium.* This was demonstrated in comparative experiments on the effect of tissues from 11 plant species on nitrogenase activity in *Rhizobium* and *Azospirillum.*[91] Nitrogenase activity was also induced by conditioned medium from cell suspension cultures of *Pennisetum americanum* and *Panicum maximum.*[108] Thus, plant cell metabolites exert a stimulating effect on the nitrogenase of *Azospirillum.* The ARA of the bacterial cells isolated from artificial association with sugarcane tissue was three to five times greater than that of the stock cultures of *A. brasilense.*[90]

Despite the possibility of a long subculturing of the association between sugarcane tissue and *A. brasilense* on medium without combined nitrogen and the presence of higher ARA in the associated bacteria, the contribution of bacterial nitrogen fixation to the growth of plant cells in the given system has not been proven. The association showed no increment of its biomass exceeding that of the biomass in the control (sugarcane monoculture on medium without combined nitrogen is capable of slow growth).[87]

Inoculation of tobacco tissue with *Azotobacter vinelandii* cultures failed to produce association.[87] Another experiment with inoculation of carrot callus with *A. vinelandii* suggested a metabolic relationship between the partners.[38] For this purpose the authors used an adenine auxotroph which grew on medium without adenine only in the presence of the callus. In turn, the callus grew on medium without combined nitrogen only in the presence of the living bacteria; the bacterial cells killed with penicillin did not maintain the growth of the callus under these conditions. Apparently, in this association the carrot cells received combined nitrogen as a result of nitrogen fixation by bacteria which, in turn, depended on the adenine of the carrot cells for their growth. The inoculated callus retained its ability to grow on medium without combined nitrogen for 18 months.

Both *Azotobacter vinelandii*[38] and *Azospirillum brasilense,*[88,89] in the respective associations, were located on the surface or in intercellular spaces of the callus tissue and never penetrated into the living plant cells.

An attempt to regenerate plants from an association of carrot callus with *Azotobacter vinelandii* proved unsuccessful.[38] In the association of sugarcane tissue and *Azospirillum brasilense,* incubated under light, green shoots were regenerated which did not differ from those obtained in the control callus culture.[87] They were capable of forming roots and growing in soil. However, neither the shoots nor the roots showed bacterial cells. The impossibility of the bacteria to penetrate the shoots in the process of organogenesis in mixed culture is generally associated with the anatomical features of sugarcane tissue.[89] This callus tissue has very dense areas which are characterized by the absence of intercellular spaces. The bacteria grow on the surface areas of callus aggregates, and this is accompanied by the death of plant cells. Due to the absence of intercellular spaces, the bacteria cannot penetrate deep into these aggregates, where viable plant tissue is preserved. Meristemoids are formed only in the tight callus areas which lack intercellular spaces. This appears to be the cause for the elimination of bacteria from tissues of the regenerated shoots or plants.

ASSOCIATIONS WITH GREEN ALGAE

Representatives of green algae are found in symbiosis with other organisms, including higher plants.[109] In his experiments on establishing artificial associations, Bradley used *Chlorella* strains capable of excreting nitrogenous compounds when grown on nitrogen-deficient medium.[94] These tests revealed the ability of green algae to delay the degradation of plant cells under conditions of deficiency in combined nitrogen.

Inoculation of carrot callus[60,93] or suspension cultures[94] with one of the *Chlorella* strains and culturing of the system on nitrogen-deficient agar medium in the light led to the survival of the callus for several months, whereas the control callus died. Algal cells grew on the callus surface and penetrated into intercellular spaces. The inoculated callus exhibited a high percentage of intact cells and most of them seemed to be alive.[61,94] The cells of another algal strain did not grow together with callus and in their presence there was a very low percentage of intact carrot cells.[94] These experiments led to the conclusion that the carrot cells are provided with nitrogenous compounds by the alga when the system is cultured on nitrogen-deficient medium.

ASSOCIATIONS WITH FUNGI

The method of coculturing *Ruta graveolens* tissue with various fungi was used as a new unconventional approach to enhancing the biosynthesis of specific products produced by plant cells *in vitro*.[95] The coculture was obtained in such a manner that the callus and the fungal mycelium did not come in contact with each other; that is, the biosynthesis was influenced by fungal excretions diffusing through the agar.

Different fungal species produced a different effect on the growth of *R. graveolens* tissue and on the biosynthesis of rutacridone-epoxides by the cells. At the same time, the fungi inhibited or stimulated the growth of the callus, as compared to the control, accompanied by an increase of specific biosynthetic activities. Culture filtrates also stimulated biosynthesis. Depending on the fungal species, the greatest stimulating effect was exerted either by mycelium or culture filtrates of the fungus. For instance, in some cases *Botrytis allii* mycelium induced a rise of acridone-epoxide content 10-fold, while the culture filtrates did so by more than 50-fold. Conversely, *Rhodotorula rubra* mycelium increased biosynthesis 30 times, while the culture filtrate increased it only twice as much compared to the control.

The addition of autoclaved fungal mycelia also induced a 72% increase in the diosgenin production in *Dioscorea deltoidea* cell culture as compared to the biosynthesis in the control culture.[110]

As a result of the experiments described here, the following facts emerge which are important for advancing the work on designing new cellular systems:

1. The possibility of obtaining active nitrogen-fixing cellular systems between partners which do not form natural associations has been demonstrated. It has also been shown that the plant cells, or metabolites secreted by them, induce or stimulate nitrogenase activity.

2. The ability to form mixed cellular systems growing on medium without combined nitrogen (apparently at the expense of bacterial nitrogen fixation) has been discovered.
3. The normal organogenesis in plant tissue (of sugarcane) in the presence of a large bacterial population has been ascertained, although the bacteria in these experiments were not incorporated into the plants.
4. A considerable increase in specific biosyntheses by plant cells, under the action of microorganisms, has been established.

Thus, the techniques developed in the experiments with various microorganisms can be deployed for producing systems similar to natural symbioses, as well as for further experimental work to improve the properties of plant cells *in vitro* or of the whole plant.

CYANOBACTERIA IN ARTIFICIAL ASSOCIATIONS WITH CULTURED CELLS, TISSUES, AND PLANTS

GENERAL STUDIES

Numerous attempts to introduce cyanobacteria into isolated protoplasts have failed to produce viable systems (Table 1). Failures in this direction can apparently be attributed to the inadequate introduction technique rather than to the biological properties of objects introduced into protoplasts. Mixed culturing or coculturing of plant cells (tissues) with various microorganism species has made it possible in many cases to produce associative interactions of the partners (Table 2). Work on introducing cyanobacteria into populations of plant cultured cells (Table 3) was started later than with other microorganisms (Table 2). These experiments (Table 3) were also preceded by work on introducing cyanobacteria into isolated protoplasts (Table 1). The first publication on mixed culturing of cyanobacteria with plant cells was in 1979.[111] With the exception of five reports,[61,94,141,142] studies on establishing artificial associations with plant cells (tissues) have been carried out by our group at the Department of Cell Physiology and Immunology of Moscow State University.

The ability for joint growth of more than 20 combinations in pairs of 7 cyanobacterial genera and 7 plant species has been studied. The plant species have been used in the form of isolated protoplasts, suspension, callus cultures, and regenerates (plants and shoots) (Table 3), as well as vegetatively propagated plants.

In experiments involving suspension and callus cultures, as well as in plant regeneration experiments, tobacco cell (tissue) cultures have often been used as a model system.

Heterotrophically growing suspension cultures of plant cells include producers of natural compounds. *D. deltoidea* synthesizes steroid compounds;[146] *P. somniferum*[147] and *Solanum laciniatum*[148] cultures are the producers of alkaloids. *Panax ginseng* is a producer of biologically active substances; this forms the basis for the industrial production of its biomass.[24]

Along with the *P. ginseng* strains auxotrophic in phytohormones, prototrophic strains have also been used.[117,118,123] Phytohormones are known to exert an inhibiting effect on nitrogen fixation in cyanobacteria.[149] The use of hormone-independent strains of plant cells would promote the establishment of nitrogen-fixing associations.

Cells of alfalfa, which is a legume adapted in the evolutionary process to symbiosis with nitrogen fixers, were also used for growing in suspension and callus mixed cultures. To obtain mixed callus cultures with subsequent regeneration of plants, besides alfalfa culture, a callus culture from a variegated tobacco mutant cv. Samsun[150] was used that was isolated from chlorophyll-deficient areas of leaf mesophyll.[136,137,139] The same plant was used to obtain isolated protoplasts.[76] The use of this mutant helped to visually identify the entry of blue-green cyanobacterial cells into the tissue of callus and regenerates which lack their own chlorophyll.

The cyanobacteria used in these experiments differed in their physiological properties.

Anabaena variabilis ATCC 29413, *A. flos-aquae, Nostoc muscorum, Anabaena* sp., *Nostoc* sp., *Chlorogloeopsis fritschii*, and *Gloeothece* are nitrogen-fixing cyanobacteria; *Calothrix elenkinii* was found to be unable to fix nitrogen. *Anacystis nidulans* and a nonnitrogen-fixing *Anabaena variabilis* (CALU-458) are obligate phototrophs. *A. variabilis* (ATCC 29413), *Chlorogloeopsis fritschii*, and *N. muscorum* are capable of growing not only in light but also in the dark. Along with a wild strain, auxotrophic biotin- and phenylalanine mutants of *Anacystis nidulans* were used. However, the dependence of their growth on the plant partner with respect to these compounds could not be ascertained.

Experiments on the introduction of cyanobacteria into plant cell populations are being carried out in three areas:

1. Studies on the effect of cyanobacteria on the growth of plant cell cultures. In this case, a deficiency of nutrition sources, primarily that of sucrose, is created. The plant cells in this event take up photosynthetic metabolites of cyanobacteria or the lysates of cyanobacterial cells. Apart from this, due to a facultative phototrophic nature, *C. fritschii* causes a delay in the degradation of ginseng cells when the system is grown in the dark.[8,118] Those systems in which the viable cells of the partners have a favorable effect on the growth of each other have been referred to as symbiotic associations.
2. Studies on the specific biosynthesis of secondary plant cell products in mixed cultures with cyanobacteria. Such systems are cultured in medium with limited carbon and nitrogen sources to establish a dependence of plant cells on the photosynthetic or nitrogen metabolism of cyanobacteria. In these cases an increase in the biosynthetic activity of plant cells in the presence of cyanobacteria is considered to be a criterion for establishing associative interactions.
3. Experiments aimed, on the one hand, at plant regeneration from mixed cultures to establish stable associations of plants with cyanobacteria and, on the other hand, at determining the possibility of the growth of such associations with nitrogen-fixing cyanobacteria under conditions of nitrogen deficiency. Mixed cultures of plant cells and cyanobacteria have also been used to study the effect of a plant partner on the nitrogen fixation of cyanobacteria and the effect of cyanobacteria on the survival and growth of plant cells (tissues).

All the above enumerated aspects of work on establishing artificial associations with cyanobacteria are considered below.

CYANOBACTERIA AS A PHOTOTROPHIC COMPONENT IN ASSOCIATION WITH PLANT CELLS

Effect of Cyanobacteria on the Growth of Plant Cells

Murashige & Skoog (MS)[151] medium, which is generally used for plant cell cultures, was chosen to check the ability for joint growth of plant cells and cyanobacteria in suspension cultures. This medium contains basic mineral compounds, many of which are incorporated in the BG-11 medium for culturing cyanobacteria.[52] In addition, it contains phytohormones necessary for the division of plant cells (and some other components) as well as sucrose (3% concentration). However, the concentration of mineral salts in the MS medium exceeds that in the BG-11 medium. Therefore, the concentration of all the components in the MS medium for producing mixed cultures was reduced by twofold[111,112] or fourfold.[117,118,123] In order to establish the interaction of the partners on the basis of photosynthetic products of cyanobacteria, the mixed cultures were grown in the light at a low concentration of sucrose (1.875 g/l). This modified MS medium was not optimal for the growth of either plant cells or cyanobacteria. The growth of different plant cell cultures on this medium without cyanobacteria (i.e., in monocultures) was two to six times lower than their growth on a full

TABLE 4
Joint Growth of Plant Cells and Cyanobacteria in Suspension Cultures[112,118,123]

Culture		Effect of joint culturing on growth	
Plant cells	**Cyanobacteria**	**Plant cells**	**Cyanobacteria**
Nicotiana tabacum	*Anacystis nidulans*[a]	+ + + +	+ + + +
	A. nidulans[b]	+ + + +	+ + + +
Panax ginseng[c]	*Chlorogloeopsis fritschii*	+ + + +	+ + + +
Dioscorea deltoidea[d]	*A. nidulans*[a]	+ + + +	+ + + +
D. deltoidea[e]	*A. nidulans*[a]	+ + + +	− − − −
D. deltoidea[f]	*A. nidulans*[a]	+ + + −	− − − −
M. sativa	*Anabaena variabilis*[g]	+ + + −	+ + + +
	C. fritschii	+ + + −	+ + + −
	Nostoc muscorum	+ + − −	+ + + −
	Calothrix elenkinii	+ + − −	+ + − −
P. ginseng[h]	*A. variabilis*[g]	+ + − −	+ + − −
	Chlorogloeopsis fritschii	+ + − −	+ + + −
	N. muscorum	+ + − −	+ + − −
	Calothrix elenkinii	+ + − −	+ + − −
Solanum laciniatum	*A. variabilis*[g]	+ + − −	+ + − −
	Chlorogloeopsis fritschii	+ + − −	+ + − −
Papaver sonniferum	*A. variabilis*[g]	− − − −	− − − −

Note: + + + + = Growth stimulation of culture; + + + − = slight growth stimulation of culture; + + − − = no effect on culture growth; − − − − = growth inhibition and death of culture.

[a] Wild strain.
[b] Auxotrophic biotin mutant.
[c] Phytohormone-auxotrophic strain.
[d] Cyanobacteria were seeded to previously growing *D. deltoidea* cells.
[e] *D. deltoidea* cells were inoculated to the previously growing cyanobacteria.
[f] Simultaneous inoculation of *D. deltoidea* cells and cyanobacteria; the remaining mixed cultures except var. d and e were produced by simultaneous inoculation of growing cells and cyanobacteria.
[g] Nitrogen-fixing strain.
[h] Phytohormone-prototrophic strain.

MS medium. Cyanobacteria in monocultures on this medium exhibited very slight growth or no growth at all.

Selection of nonoptimal conditions for either of the partners in coculturing was justified by the fact that (1) these conditions provided no advantages for the development of any of the partners and (2) in the case of their favorable effect on each other, even slight interactions can be ascertained under conditions of nonoptimal growth.

Coculturing helped ascertain the species specificity in the interaction of partners in pairs (Table 4). This was manifested by the fact that plant cells and cyanobacteria produced different effects on each other. There was mutual growth inhibition of *Papaver somniferum* and *Anabaena variabilis* (Figure 2). The dry weight in the mixed culture did not increase, and on day 12 of culturing it had only 10% of living cells of *P. somniferum*. The monoculture of *P. somniferum*, however, contained 60 to 62% of living cells and their dry weight rose 4.5 times during this period (Figure 2A). *A. variabilis* cultured together with *P. somniferum* cells did not grow and its cells lost pigment and died off (Figure 2B).

In most cases, no effect, or a slight stimulating/depressing effect, of one partner or the other was detected[123] (Table 4). However, cyanobacteria were found to stimulate the growth of plant cells in tobacco,[111,112] one of the ginseng strains,[117,118] and *D. deltoidea* cells in two culture versions (Table 4). The systems in which cyanobacteria were found to have a stimulating effect, or no inhibiting influence, on the growth of plant cells were of greatest interest.

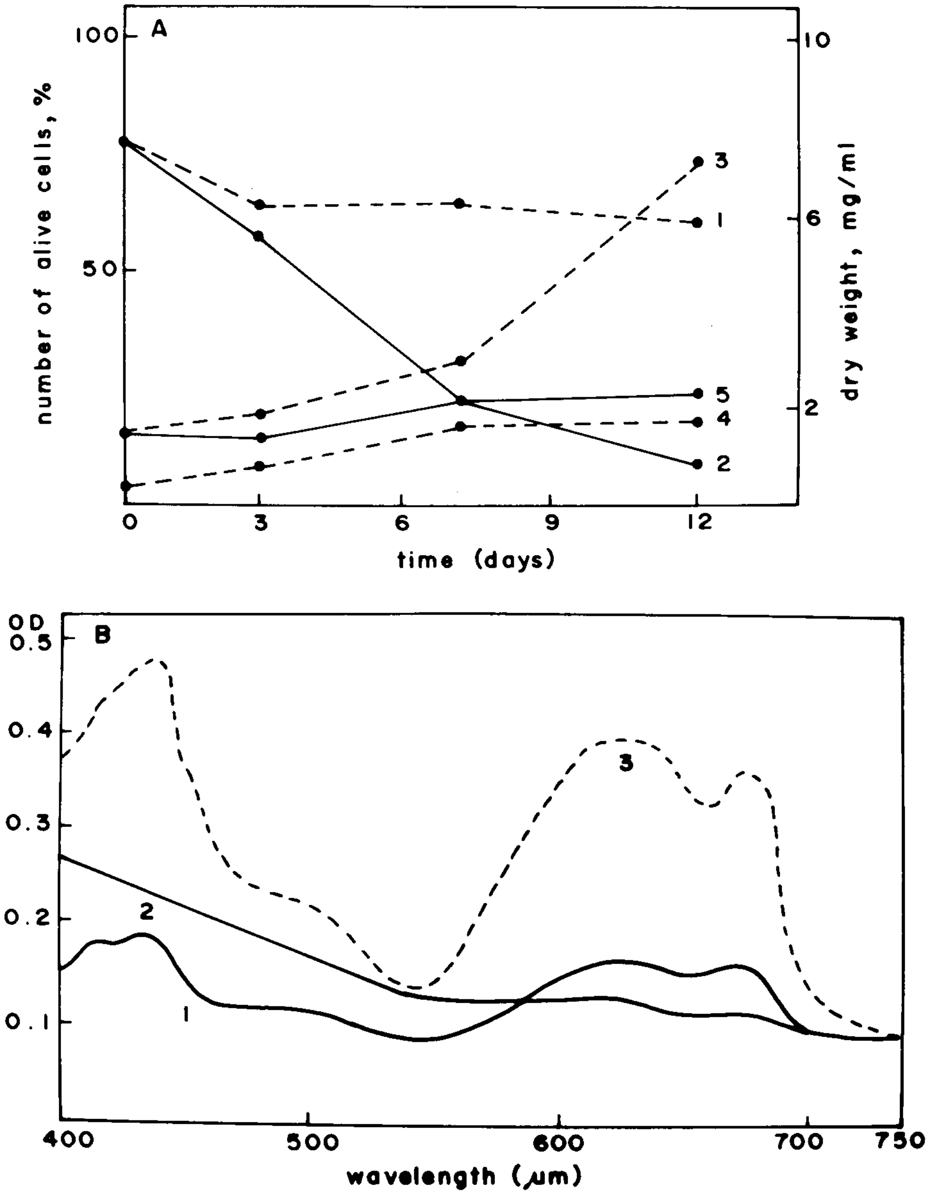

FIGURE 2. Growth of mono- and mixed cell cultures of *Papaver somniferum* and *Anabaena variabilis*[123] on $^{1}/_{4}$ MS medium with $^{1}/_{16}$ N sucrose content. (A) Changes in the number of living cells of *P. somniferum* in (1) mono- and (2) mixed cultures, in the dry weight in monocultures of (3) *P. somniferum* and (4) *A. variabilis*, and in their (5) mixed culture. (B) Absorption spectra of mixed *P. somniferum* and *A. variabilis* cell culture suspensions at (1) the initial stage and (2) on day 3 of culturing; (3) *A. variabilis* monoculture on day 3 of culturing.

Tobacco and *Anacystis nidulans* cells cause a joint stimulation of their growth (Figure 3). The growth peak of tobacco cells follows that of cyanobacteria. The establishment of joint growth stimulation requires a considerable decrease of sucrose content in the medium. For instance, the growth of a mixed culture of ginseng cells (auxotrophic strain) and *C. fritschii*, when sucrose concentration was decreased eightfold ($^{1}/_{8}$ N), did not exceed the summed up growth of the respective monocultures (Figure 4). The subsequent decrease in sucrose concentration ($^{1}/_{16}$ N) led to the the mixed culture growth which was twofold higher than the total growth of the corresponding monocultures.

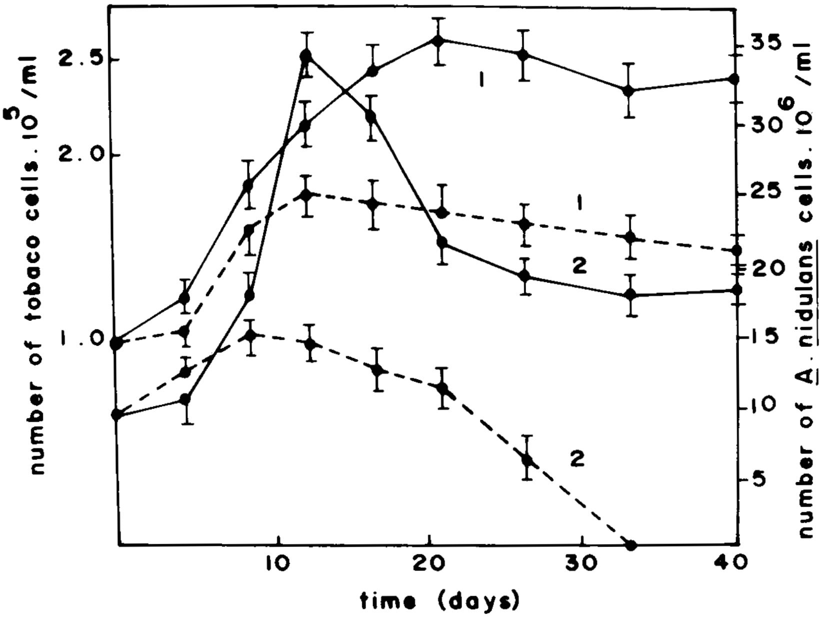

FIGURE 3. Mutual growth stimulation of (1) tobacco cells and (2) *Anacystis nidulans* in mixed culture (solid line).[8,114] Dotted lines designate growth of the corresponding monocultures. ($^1/_2$ MS medium, $^1/_{16}$ N sucrose; light intensity 750 lx).

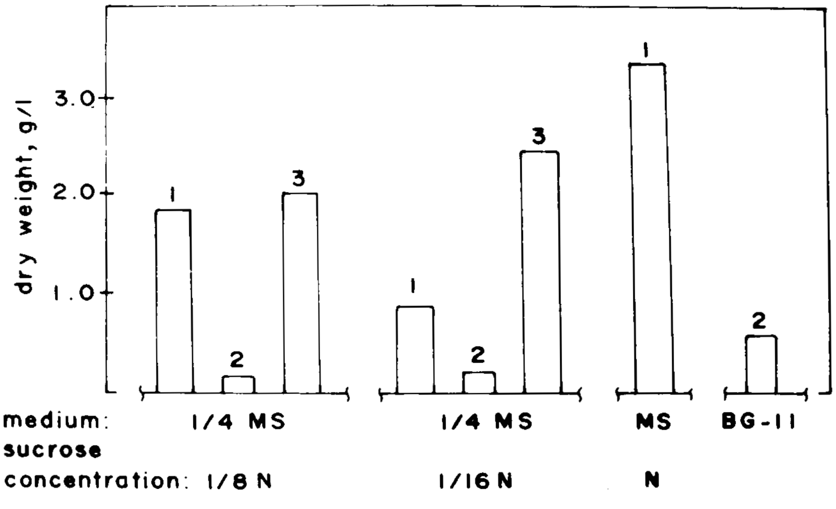

FIGURE 4. Growth of (1) ginseng cells and (2) *Chlorogloeopsis fritschii* in monocultures and (3) in mixed culture in the light.[8,118] (Light intensity 750 lx; growth period 14 d.)

The results of experiments summarized in Table 4 reveal the species and strain specificity in the interaction of partners. The associative interactions in artificial systems, as well as natural symbiotic associations,[12,53,152] are formed under unfavorable conditions of growth of either partner. The character of interrelations between the partners may depend upon different factors. For example, in one pair (cells of *D. deltoidea-A. nidulans*) the relations of the partners depend upon the method of their inoculation (Table 4). This is apparently connected with conditioning the medium by one partner and creation of more favorable conditions for the growth of the other partner. Apart from this, the formation of associative interactions

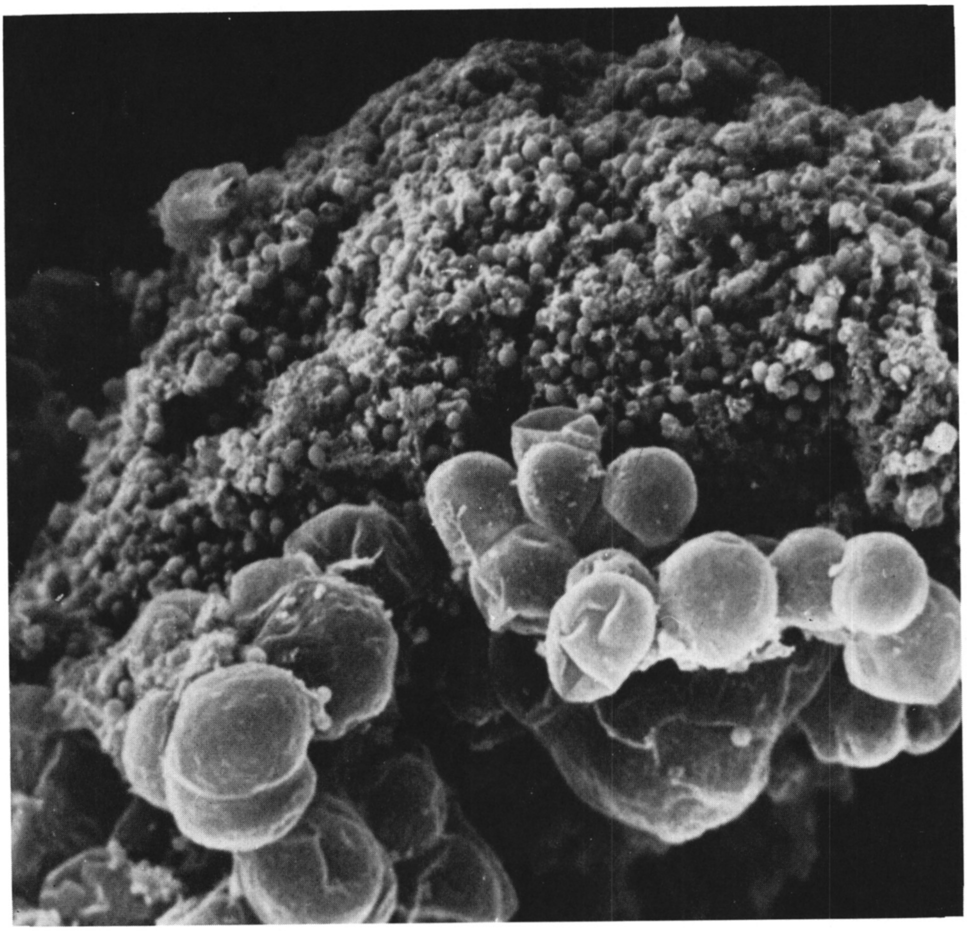

FIGURE 5. Clusters of *Chlorogloeopsis fritschii* cells on the surface of ginseng cell aggregates (culture version c according to Table 4). (SEM; magnification × 300).

are influenced by secondary products of plant cells. For example, cells of *S. laciniatum* and *P. somniferum* produce alkaloids, but the former accumulate them intracellularly[148] while the latter excrete them into the medium.[147] This probably results in an inhibiting effect of *P. somniferum* on *Anabaena variabilis*, while *S. laciniatum* cells do not exert such an effect.

Cellular Contacts

Plant cells in mixed cultures can grow with cyanobacteria in close contact or without contact. Some *C. fritschii* cells are always adsorbed on the surface (Figure 5) and penetrate deep into ginseng aggregates. The number of ginseng aggregates forming contacts with *C. fritschii* increases when sucrose is excluded from the medium.[128]

A. variabilis (Figure 6) and *C. fritschii* (Figure 7) also form contacts with the surface of the aggregates, and single living cells of *S. laciniatum* and penetrate deep into the aggregates. *Anacystis nidulans* cells form mixed aggregates with tobacco cells when exposed to low illumination (500 to 750 lx) (Figure 8), penetrating through intercellular spaces deep into the aggregates (Figure 9). This cyanobacterium does not come into contact with tobacco cells when exposed to more intense illumination (1500 to 2000 lx).[112] In the case of mutually beneficial growth of *D. deltoidea* cells with the same cyanobacteria (Table 4), no mixed aggregates were observed.

In all the cases, cyanobacteria were adsorbed predominantly on the aggregates of living

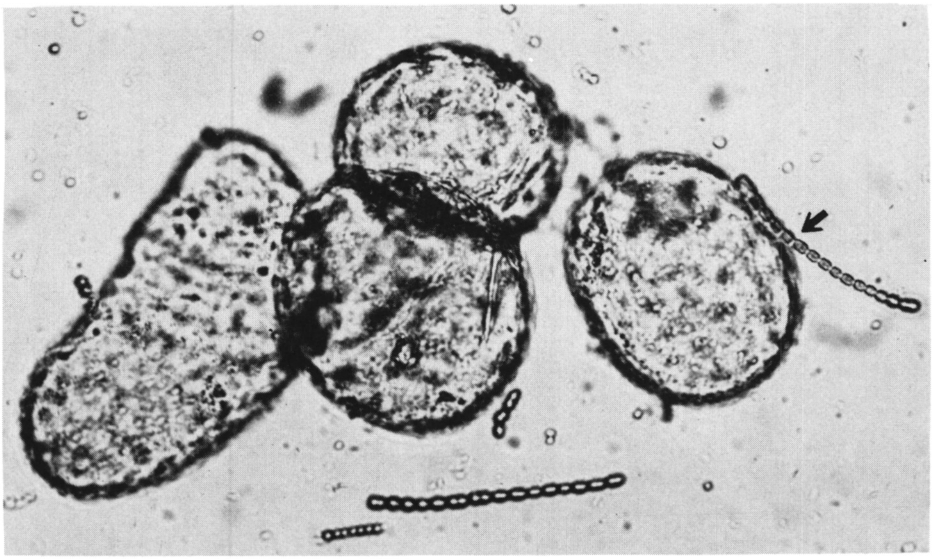

FIGURE 6. Contact of a cyanobacterium, *Anabaena variabilis* (indicated by arrow), with a *Solanum laciniatum* cell.[133] (Light microscopy; magnification × 500.)

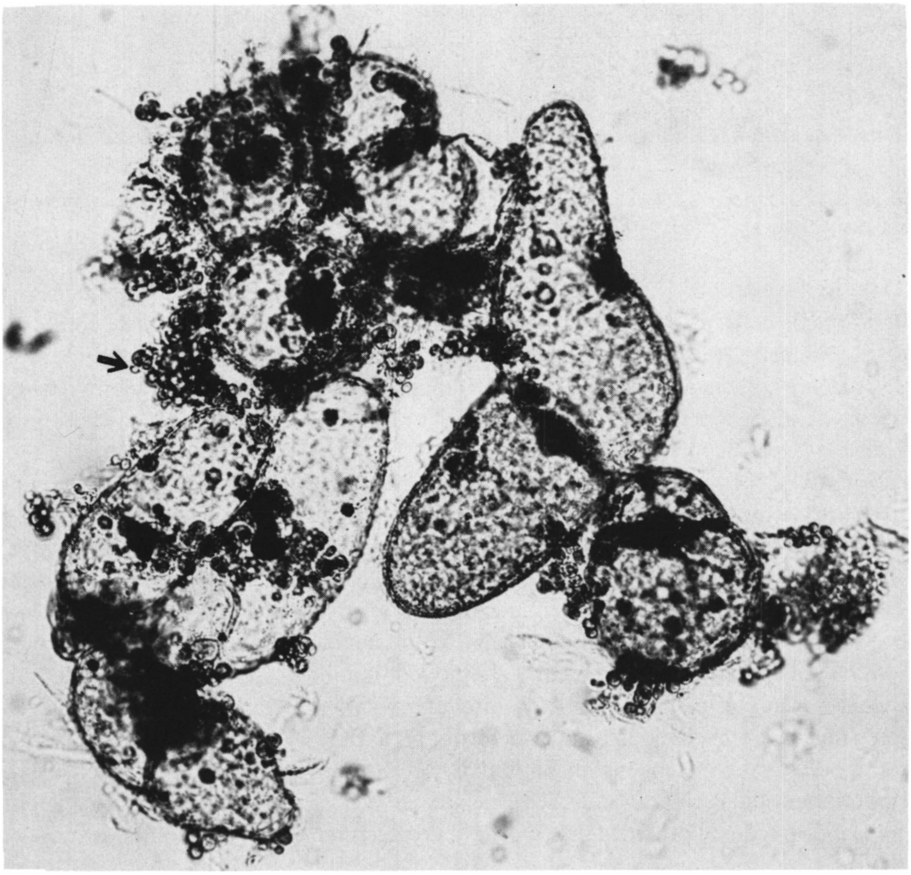

FIGURE 7. Mixed aggregate of *Solanum laciniatum* and *Chlorogloeopsis fritschii* cells (cyanobacteria indicated by arrow).[133] (Light microscopy; magnification × 400).

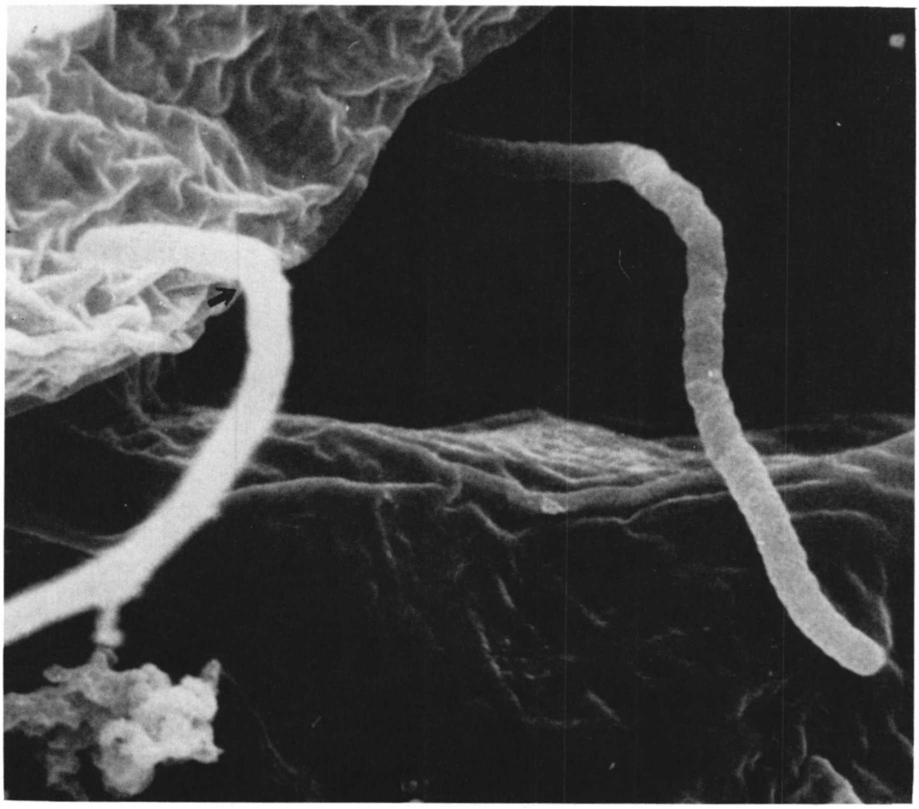

FIGURE 8. Localization of *Anacystis nidulans* in the recess between the cells of tobacco aggregate. (SEM; magnification × 5000.)

cells. Only in the event of ginseng culture (prototrophic strain) were there aggregates of *C. fritschii* on dead cells.[123] This may attest to the effect of biologically active compounds produced by ginseng cells on cyanobacteria. Subculturing of tobacco-*A. nidulans* and ginseng-*C. fritschii* associations,[11] as well as prolonged culturing (up to 5 months) without transfers of the latter system, point to the survival of the cells of the partners in the mixed aggregates.

Thus, most of the suspension cultures exhibited the formation of mixed growth zones. These zones obviously form special conditions promoting metabolic interactions of the partners and protecting the partners from the conditions of the medium, which is initially nonoptimal or even unfavorable for their development. The formation of mixed aggregates leads to the effect which is similar to the buffering effect of liverwort on a symbiotic cyanobacterium.[153] The latter acquires an ability, in symbiosis, to fix nitrogen at low pH values which are unfavorable for growth and nitrogen fixation in free-living cyanobacteria.[15] In some cases (ginseng cells-*C. fritschii*), in the zones where cyanobacteria are located on the aggregates, a homogeneous structure forms as a film ("mantle") which apparently consists of polysaccharide mucus. It is similar to mucus in the areas of contact between cyanobacteria and plant cells in natural associations.[154-156] The mantle envelops cyanobacterial cells and obviously provides the structural connections of the association components (Figure 10).

Transfer of Photosynthate from Cyanobacteria to Plant Cells

Stimulation of plant cell growth in light (when cyanobacteria grow intensively) under conditions of sucrose deficiency (as compared to the respective monocultures, Figure 3),

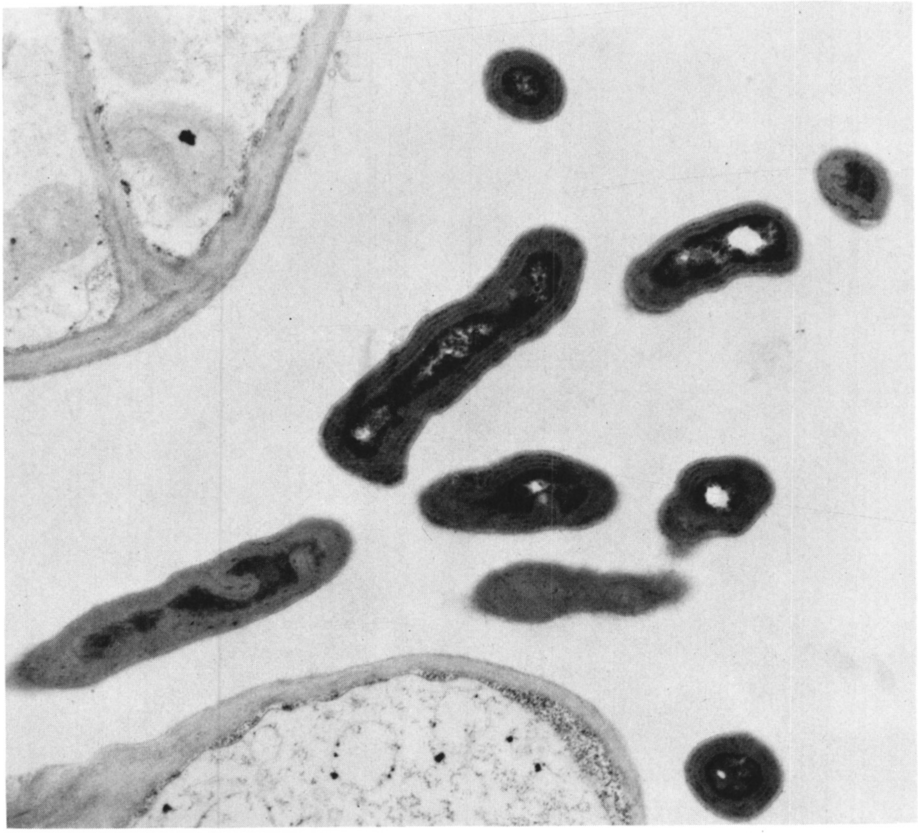

FIGURE 9. *Anacystis nidulans* in the intercellular space of a tobacco aggregate. (TEM; magnification × 13,500.)

the formation of contacts by the partner cells, and the dependence of this process on the presence of sucrose and light provide evidence for the transfer of photosynthetic products from cyanobacteria to plant cells. Without contacts, the cells apparently interact by way of transferring the photosynthetic products through the medium.[112]

The use of ^{14}C-labeled bicarbonate showed that *C. fritschii*, when grown in the light on BG-11 medium, was capable of excreting into the medium up to 16% of ^{14}C fixed in the photosynthetic process (Table 5). Incubation of ginseng cells in this culture medium, after *C. fritschii* had been removed, led to the incorporation of the label present in the medium into the ginseng cells. No incorporation of labeled metabolites into dead ginseng cells was observed.

A cultural liquid of *C. fritschii*, added in the proportion 3:1 to the full MS medium free of sucrose, supported the growth of ginseng cells comparable to that in fourfold diluted MS medium with sucrose (in a concentration of about 1%).[121] The ability of plant cells to take up the photosynthetic products of cyanobacteria has thus been ascertained. The published data show that cultured cells can take up a wide range of carbon sources (various hexoses and polysaccharides).[157]

Uptake of Cyanobacterial Lysates by Plant Cells

Studies on the growth dynamics of mixed culture show (Figure 3) that, up to a certain time, the growth of tobacco cells exceeded their growth in monocultures and was accompanied by active multiplication of cyanobacteria which provided them with photosynthetic

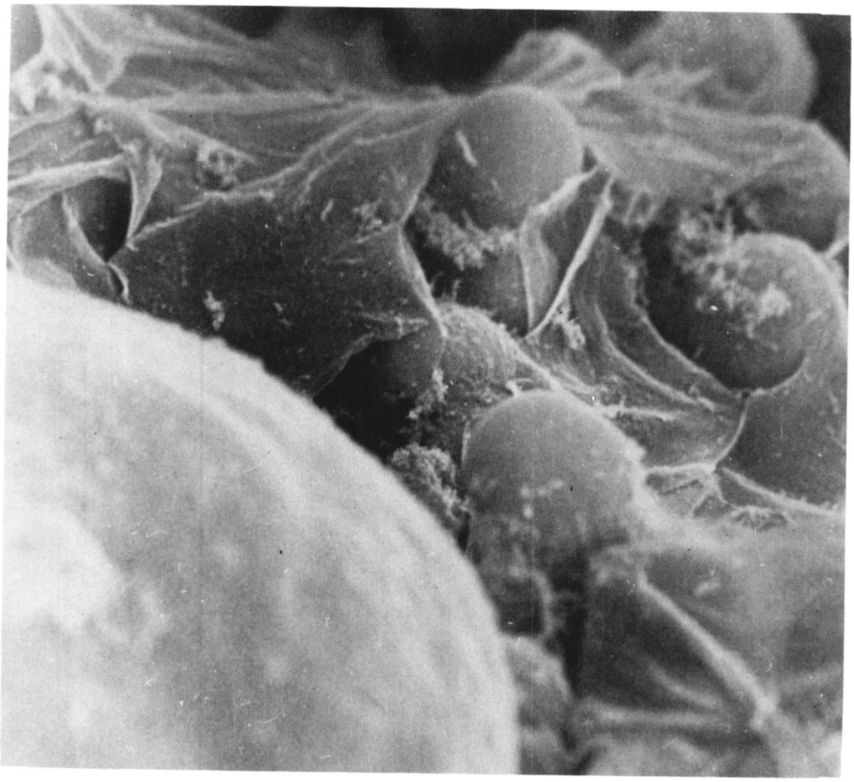

FIGURE 10. Homogeneous structure — "mantle" — surrounding *Chlorogloeopsis fritschii* cells on the surface of a ginseng cell aggregate (culture version c Table 4. SEM; magnification × 5,000.)

TABLE 5
Excretion by Cyanobacteria and Uptake by Ginseng Cells of NaH^{14}CO$_3$ Photosynthetic Fixation Products of *Chlorogloeopsis fritschii*[121]

Culture[a]	Sample	^{14}C (mg/l/g dry weight per day)
Chlorogloeopsis fritschii	Cells	147.8 ± 6.78
	Culture liquid	26.4 ± 2.17[b]
Panax ginseng	Living cells	19.6 ± 3.35
	Heat-killed cells	0.37 ± 0.094

[a] *C. fritschii* were incubated for 24 h in BG-11 medium in the presence of NaH ^{14}CO$_3$ (illumination intensity 4000 lx). The cells were then isolated from the culture liquid and ^{14}C was determined in the cells and in the culture medium. Next, ginseng cells were placed into the culture medium of *C. fritschii* and, after a 24-h incubation, the ginseng cells were assayed for ^{14}C.

[b] mg/l/d excreted by *C. fritschii* cells corresponding to 1 g dry weight.

products. This was supported by experiments with other mixed cultures.[121] Apart from this, the subsequent growth of the cultured tobacco cells, exceeding their growth in monoculture, in the second growth phase of the system was accompanied with a considerable lysis of cyanobacterial cells (Figure 3). In another system (*D. deltoidea* cells-*A. nidulans*, culture version according to Table 4), it was shown that cyanobacteria did not multiply in mixed

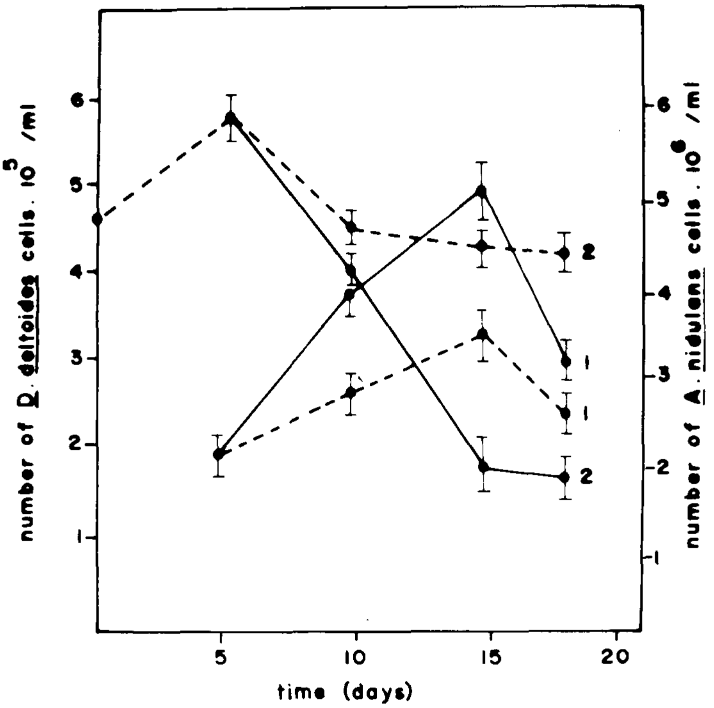

FIGURE 11. Growth of (1) *Dioscorea deltoidea* and (2) *Anacystis nidulans* cells in mixed culture (solid line). The *D. deltoidea* cells were inoculated to growing cyanobacterial cells. Dotted line designates growth of the corresponding monocultures.

culture and were intensively lysed. The growth of *D. deltoidea* cells in the presence of cyanobacteria exceeded that in monoculture (Figure 11). These experiments lead to the conclusion that the growth of plant cells may be supported by cyanobacterial lysates.

The lysis of cyanobacteria in mixed culture was more rapid than in the respective monocultures. This process may result either from autolysis or lysis induced by plant cells. Cyanobacteria are known to possess autolytic activity.[158] However, it is more probable that plant cells have a lytic effect on cynobacterial cells. Plant callus tissues are known to have bacteriolytic activity arising from the presence of endogenic lysozyme.[159]

In some natural symbiotic systems, in analogy with the obtained experimental systems of tobacco and *D. deltoidea* with *A. nidulans*, the symbiont can utilize the products of the photosynthesis and partial lysis of algae.[53] Thus, the tests of the ability for a joint growth in a great number of combinations made it possible to select the pairs in which cyanobacteria had a favorable effect on the growth of plant cells. The associations were formed under conditions which were nonoptimal for either partner. Carbon-deficient media exhibited the ability of plant cells to assimilate the photosynthetic or lytic products of cyanobacteria. The latter fact can be important for the use of plant cells in biotechnology for replacement of sucrose, used for food purposes, by less-deficient sugars. A search for such substrates is currently underway. This includes studies on the possibility of cultured plant cells to grow on such unusual substrates as syrup, lactose, and whey (which may be present in waste products of sugar refineries and dairy plants).[157] Cyanobacterial lysates can be one such untraditional source of nutrition for plant cells. Their production from mass cyanobacterial culture and their use in growing plant cells on an industrial scale may prove profitable.

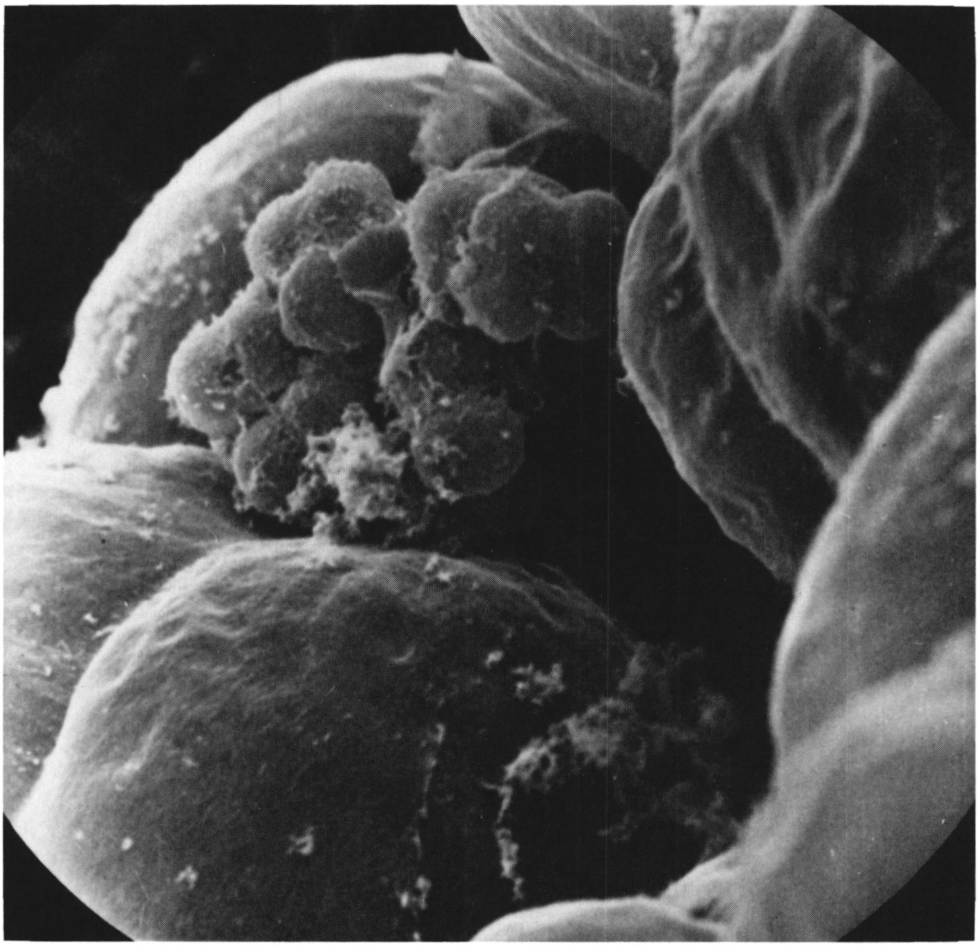

FIGURE 12. The mixed aggregate of ginseng and *Chlorogloeopsis fritschii* under the conditions of culturing in the dark. (SEM; magnification × 3,700.)

BIOSYNTHETIC ACTIVITY OF PLANT CELLS IN MIXED CULTURES WITH CYANOBACTERIA

The ability of cell cultures in species-specific biosynthesis of secondary products has been tested in associations with cyanobacteria.

Synthesis of Steroids

D. deltoidea, in mixed cultures with *A. nidulans* grown in the light on sugar-deficient ($^1/_{16}$ N) modified medium ($^1/_2$ MS), synthesized up to 80% of steroid compounds formed in monoculture on full medium.

Associations of ginseng (phytohormone-auxotrophic strain) and *S. laciniatum* cells with *C. fritschii*, besides growing in the light (Table 4), can be grown in the dark, due to the ability of the given cyanobacterium for heterotrophic growth. These systems were cultured in the dark on MS medium diluted fourfold ($^1/_4$ MS) with a twofold decrease of sucrose concentration as compared to the full medium (i.e., up to $^1/_2$ of N).

Synthesis of Biologically Active Compounds by Ginseng Cells and Their Effect on *C. fritschii*

When grown in the dark, ginseng cells-*C. fritschii* associations (Figure 12) are formed at a higher sucrose concentration ($^1/_2$ N, Figure 13) than in the light ($^1/_{16}$ N, Figure 4). This

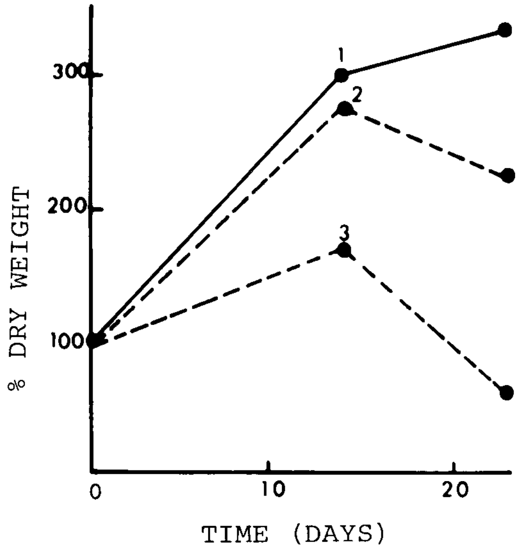

FIGURE 13. Delay of ginseng cell degradation in mixed culture with *Chlorogloeopsis fritschii* grown in the dark.[8,118] (1) Mixed culture; (2) and (3) corresponding monocultures of ginseng cells and *C. fritschii.* ($^1/_4$ MS medium; $^1/_2$ N sucrose.) 100% of dry biomass corresponds to the initial inoculation of ginseng culture (0.95 g/l) and that of cyanobacteria (0.17 g/l).

stems from the sucrose requirements of the cyanobacterium for its growth in the dark. In monocultures grown in the dark, the partners were lysed by the 2nd week of culturing; in mixed culture they continued to grow during this time.

Ginseng cells in culture are known to retain their ability for biosynthesis of biologically active substances (ginsenosides) belonging to triterpenoid glycosides.[160,161] In mixed culture with *C. fritschii*, the ginseng cells synthesize biologically active substances. The total content of glycosides in mixed culture accounts for 5 to 7% of the biomass of the dried ginseng cells, as in the monoculture grown on a full MS medium.[122] Thus, despite a decrease in the concentration of the medium constituents, ginseng cells growing together with *C. fritschii* retain the level of their biosynthetic activity.

Special tests demonstrated that when BG-11 medium is supplemented with ginseng cell extract (water-alcohol extract) to a final concentration of 10%, there is a fivefold increase in endogenous oxygen consumption as compared to the control (Figure 14). The use of extracts at different concentrations failed to reveal their influence on the photosynthetic evolution of O_2 by *C. fritschii*. The observed dependence of the increase in the respiration intensity of *C. fritschii* coincides with the previously noted effects of ginseng root extract on the respiration of *Escherichia coli.*[162]

In studies of cellular interactions in ginseng cells and cyanobacterial associations, it seems important to take into account the effect of biologically active substances of ginseng on the metabolic activity of *C. fritschii*. A biochemical analysis of our ginseng culture pointed to the presence of these substances in the cells, but not in the culture medium.[161] A ginseng cell population always has some fraction of dead cells. During their lysis, the biologically active substances contained in the cells find their way into the incubation medium. Apart from this, the biologically active substances can influence *C. fritschii* cells occurring in direct contact with dead cells[123] or in the zones of ginseng cell destruction on the surface of the aggregates.[125] A possible influence of biologically active substances can

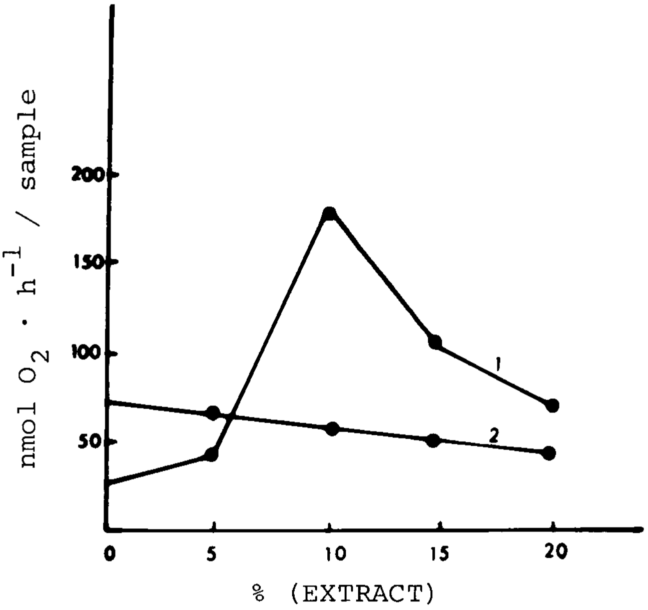

FIGURE 14. The effect of ginseng cell extract on (1) respiration and (2) photosynthesis of *Chlorogloeopsis fritschii*.[122] Ginseng cell extract, in 20% alcohol (1:10, w/v), was evaporated, diluted in distilled water, and added in designated concentrations to *C. fritschii* suspension in BG-11 medium at optical density of 2.0 units. Oxygen consumption (in the dark) and photosynthetic oxygen evolution (light intensity 25,000 to 30,000 lx) were determined polarographically.

explain the growth of *C. fritschii* in mixed culture with ginseng cells, with no growth observed in the monoculture.[118]

Stimulatory Effects of *C. fritschii* on Biosynthetic Activity of *S. laciniatum* Cells

Joint culturing of *S. laciniatum* and *C. fritschii* cells on sugar-deficient ($^1/_2$ *N*) $^1/_4$ MS medium in the dark offered no advantages for either partner. The growth of *S. laciniatum* cells both in mono- and mixed cultures was lower than that of the monoculture on a full MS medium.[133] *C. fritschii* in mono- and mixtured cultures did not grow and died in the 3rd week of culturing. Nevertheless, in the mixed culture there was a considerable increase in the content of sterols and alkaloids in *S. laciniatum* cells as compared to their accumulation in the monoculture on full medium (Figure 15).

Because the given systems used the cyanobacterium *C. fritschii* capable of nitrogen fixation, it seemed important to elucidate the possibility of mixed culturing and determine the productivity of plant cells on nitrogen-free medium. On a specially selected nitrogen-free medium, but with a sucrose content increased to 60 g/l, *C. fritschii* promotes the survival of *S. laciniatum* cells and stimulates their division; on the 10th d of culturing the mixed culture had twice as many plant cells as in the monoculture.[133] Analysis of the steroid compounds in *S. laciniatum* cells showed that mixed culturing with *C. fritschii* did not substantially change the dynamics of sterol accumulation (Figure 16A).

A comparison of the accumulation of alkaloids, depending on the culture period, showed that during initial growth periods there was a considerable increase in the biosynthesis of alkaloids in *S. laciniatum* cells in the mixed culture (Figure 16B). The accumulation curve was characteristic for a given suspension culture, with maximum alkaloid content on the 2nd day of culturing. Such an increase in the accumulation of alkaloids in *S. laciniatum*

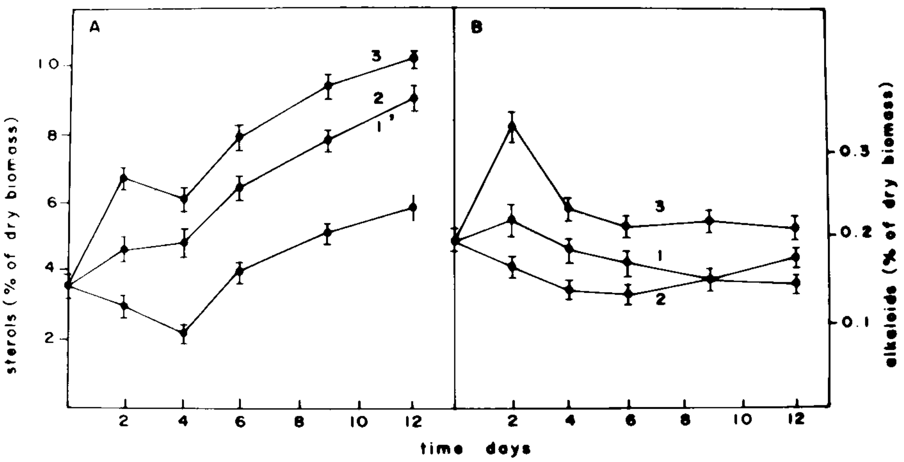

FIGURE 15. Accumulation of (A) sterols and (B) alkaloids in *Solanum laciniatum* cell culture.[133] (1) Monoculture in MS medium; (2) monoculture in *Chlorogloeopsis fritschii* culture liquid plus 1/4 MS medium with 1/2 N sucrose; (3) mixed culture of *S. laciniatum* and *C. fritschii* cells in the same medium.

cells may point to the establishment of a metabolic relation with *C. fritschii*. This attests to the fact that the plant cells are provided with nitrogen-containing compounds formed by cyanobacteria when grown on nitrogen-free media. Detection of heterocysts in the mixed culture further supported this view.

Efficiency of Mixed Culturing

The increase in the biosynthesis of sterols and alkaloids (per unit of biomass) in mixed cultures of *S. laciniatum* grown on sucrose-deficient (1/2 N) 1/4 MS medium, as compared to the biosynthesis on full medium in monoculture, posed the question of whether the mixed culturing is more profitable than the growth of monoculture on full medium. In this connection the efficiency (in terms of medium consumption) of obtaining alkaloids in mixed culture has been assessed.

For this purpose the total biosynthesis of alkaloids was calculated per unit of medium volume for both culture methods (in monoculture on full medium and mixed culture on deficient medium) on the 2nd and 12th days (Table 6). For this, the amount of alkaloids in a biomass unit (Figure 15) was multiplied by the amount of biomass grown in this volume (1 l) (Table 6). The total amount of alkaloids produced in 1 l of monoculture on the 2nd day was then taken as a unit. The total accumulation of alkaloids in the monoculture on the 12th day and in the mixed culture on the 2nd and 12th day was expressed in relative units of product formed in 1 l of culture. Finally, considering the actual consumption of MS medium and sucrose on full (N) and deficient (1/4 MS, 1/2 N of sucrose) media, the consumption of MS medium and sucrose (in parts of N) for obtaining a product unit was determined. It was found that obtaining a product unit of alkaloids in mixed culture, on the 2nd and 12th days is more profitable than in monoculture on the 12th day. It is noteworthy that practically the same amount of product can be obtained in 1 l of medium (27 mg) in monoculture on the 12th day and in 2 l of medium (26 mg) in mixed culture on the 2nd day. At the same time the consumption for producing 2 l of mixed culture will be even lower than for producing 1 l of monoculture on full medium. Thus, at about the same consumption for obtaining the same amount of product, provided that the volume of mixed culture increases twofold, a sixfold saving of the culture period can be effected. By using the same volume of mixed culture, but performing two growth cycles (i.e., twice for 2 d), a threefold saving of the culture period is obtained at somewhat lower consumption for producing the same amount of alkaloids as in the monoculture (on the 12th day).

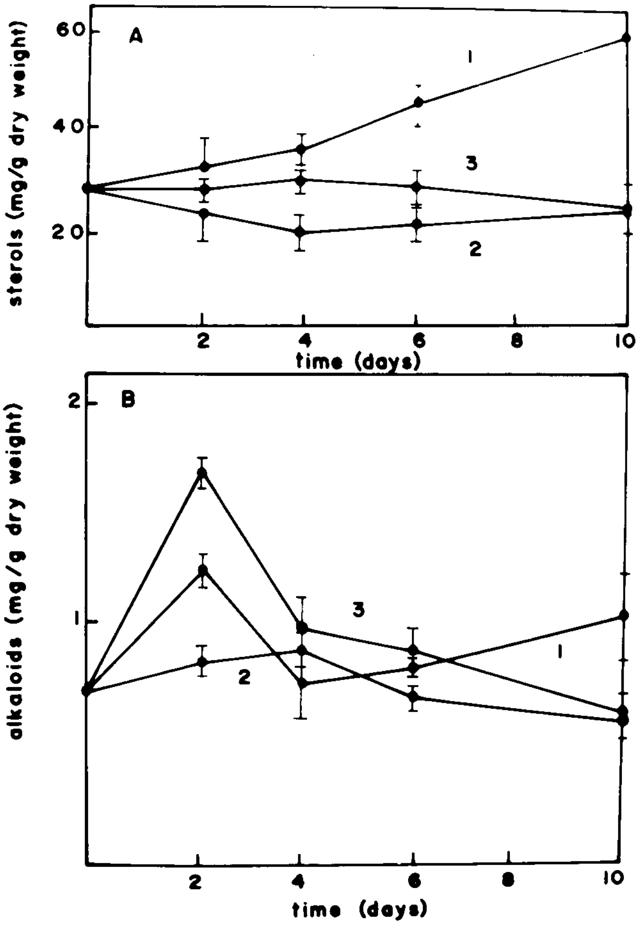

FIGURE 16. Accumulation of (A) sterols and (B) alkaloids by *Solanum laciniatum* cells.[133] (1) Monoculture in MS medium; (2) monoculture in modified nitrogen-free Allen and Arnon medium[163] (with added vitamins, mesoinositol, and phytohormones in compliance with a standard MS medium and 60 g/l sucrose); (3) mixed culture with *Chlorogloeopsis fritschii* in the same medium.

The saving of raw materials for obtaining a product unit can be important for large-scale production and offer high production efficiency. On the other hand, the systems with a high product yield at early culture periods may be profitable under continuous culturing, the introduction of which, in biotechnology, presently poses one of the most challenging problems.[21]

ARTIFICIAL NITROGEN-FIXING ASSOCIATIONS

The above experiments provide information on the use of nitrogen-fixing cyanobacteria in associations with cultured plant cells. In several, attempts were made to establish interactions for nitrogenous metabolites of cyanobiont, with combined nitrogen being excluded from the medium (Figure 16).[133] This case demonstrates advantages gained by plant cells in the presence of a cyanobiont.

To establish nitrogen-fixing associations with cyanobacteria, use has been made of suspension and callus cultures as well as inoculation of plant cuttings and regeneration of plants from mixed cultures.

TABLE 6
Consumption of MS Medium and Sucrose for Obtaining a Product Unit of Alkaloids in *Solanum lactiniatum* Cell Culture for Various Culture Methods

			Alkaloid content			Consumption per product unit of alkaloids[a]	
				In 1 l of culture			
Cell culture version	**Culture period (d)**	**Biomass (g/l)**	**Dry weight %**	**mg**	**Relative units**	**MS medium**	**Sucrose**
Monoculture	2	4	0.21	8	1	1	1
MS medium, sucrose 30 g/l (N)	12	16	0.17	27	3.4	0.3	0.3
Mixed culture	2	4	0.33	13	1.6	0.16	0.31
$^1/_4$ MS medium, sucrose 15 g/l ($^1/_2$ N)	12	8	0.22	18	2.2	0.11	0.23

[a] In parts of normal concentration in full medium.

Production of Mixed Cultures

In his experiments, Bradley[61,94] mixed carrot cell suspension culture with nitrogen-fixing cyanobacteria *Gloeothece* sp., *Plectonema boryanum,* or *Anabaena flos-aquae* and seeded on agarized B5 medium[164] with varying content of nitrogen or without nitrogen: *Gloeothece* sp. did not grow; *Anabaena* and *Plectonema*, however, showed growth in the presence of carrot cells (and the presence of callus promoted their growth). Some cells of *Plectonema* and *Anabaena* were detected in carrot cells which looked intact on the histological sections. According to the author, the cells were nevertheless destroyed, but this was not apparent on the section. The cyanobacteria were also found in destroyed cells, intercellular spaces, and on the outer surface of the callus. Latex beads used in a control version distributed throughout the intercellular spaces and on the outer surface of the callus.

Nostoc and *Anabaena* isolated in rice fields of Vietnam have been used for coculturing with tobacco callus.[141,142] When seeded jointly, the cyanobacteria exhibited a varying ability to grow on medium and in the callus. In some cases, the cyanobacteria showed an intense growth on the medium and in the callus; in the latter case the growth was manifested on the surface of the callus and in the intercellular spaces. One of the cyanobacterial strains retained its ability for photosynthesis when cultured with the callus for 1 year. The association with cyanobacteria was maintained by subculturing for 20 months.

The nitrogen-fixing cyanobacteria *A. variabilis* ATCC 29413 was used to inoculate alfalfa suspension and callus cultures isolated from leaf mesophyll.[130] The cultures were grown on B5 medium free from combined nitrogen. The association of alfalfa callus with *A. variabilis* was obtained by two methods. In one case, a mixed culture, the cyanobacteria were applied to the surface of the callus and the partners grew in direct contact with each other (the cyanobacteria grew on the surface of the callus and in the intercellular spaces). In the other case, the callus and cyanobacteria grew at some distance from each other; there was no contact between the partners (coculture).

Mixed cultures of tobacco tissue with various strains of *A. variabilis* were produced by two methods. In one case, the callus association was established by using isolated tobacco protoplasts. The protoplasts together with a nonnitrogen-fixing *A. variabilis* CALU-458 were treated with calcium ions[76] and as a result the cyanobacteria adhered to the protoplasts (Figure 17). The obtained mixture of the protoplasts and cyanobacteria was seeded on MS medium with an osmotic stabilizer; this led to the formation of mixed callus culture upon subsequent growing in the light.[77]

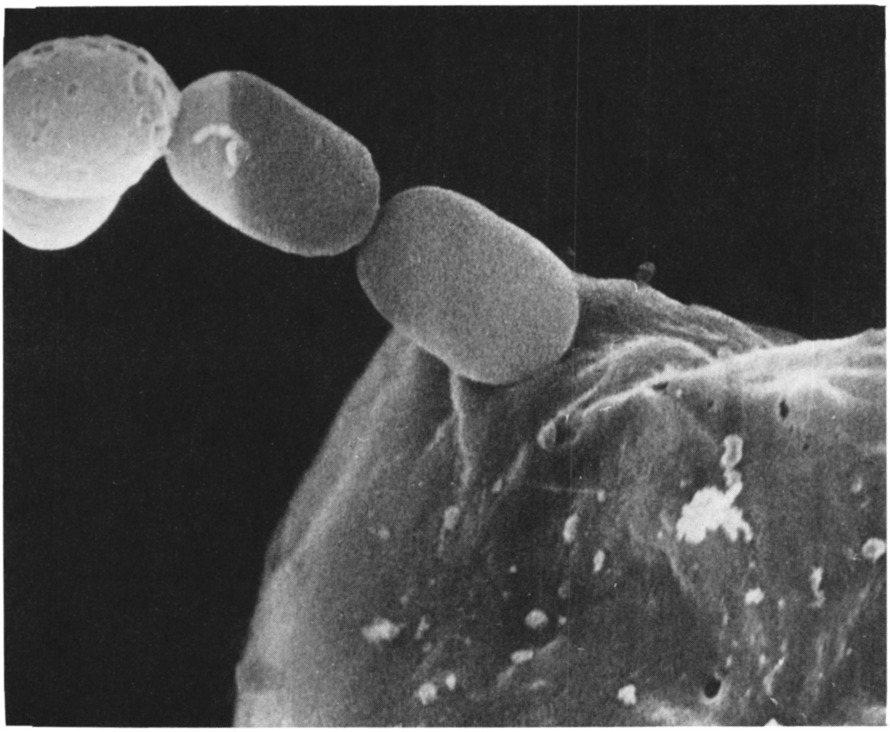

FIGURE 17. Adsorption of *Anabaena variabilis* filament (nonnitrogen-fixing strain) on the surface of a tobacco protoplast.[76] (SEM; magnification × 12,500.)

The other case involved the use of callus isolated directly from leaf mesophyll, where a suspension of nitrogen-fixing *A. variabilis* ATCC 29413 was seeded. The nonnitrogen-fixing strain of *A. variabilis* was used for obtaining a model system (mixed culture with tobacco callus and subsequent regeneration by inducing organogenesis in the callus culture). The mixed cultures and regenerates produced with various strains of *A. variabilis* were found to have both common properties and substantial differences.

In either case, the growth on MS medium in the light gave rise to callus cultures, the blue-green color of which was due to the presence of cyanobacteria (Figure 18). In these experiments combined nitrogen was present in the medium. The cyanobacteria grew on the surface of the callus (Figure 19) or penetrated deep into the tissue through the intercellular spaces (Figure 20). Infrequently, the cyanobacteria were found inside the cells, apparently lysing the callus cell walls in the places of contact with them.[140] In the callus produced from tobacco protoplasts, some cyanobacteria were located intracellularly in the central vacuoles (Figure 21). This provides evidence for the penetration of cyanobacteria into the plant protoplasts when treated with calcium ions and the preservation of their intracellular location during multiplication of the callus cells. Studies on the localization of cyanobacteria in callus samples showed that the specific feature of tobacco cells was the presence of large vacuoles and poor development of their cytoplasm at the stationary phase of culture growth. It is possible that this explains the absence of cyanobacteria in the cell cytoplasm, as distinct from location of endosymbiotic cyanelles in the cytoplasm of the host cells in natural associations.[44]

Mixed tobacco callus cultures with both strains of *A. variabilis* are stable and capable of growing during prolonged subculturing for at least 2 years.[139] The establishment of a stable mixed culture depends upon the quantitative ratio of the partner biomass; otherwise, one partner is likely to inhibit the growth of the other.[138] However, a long subculturing of

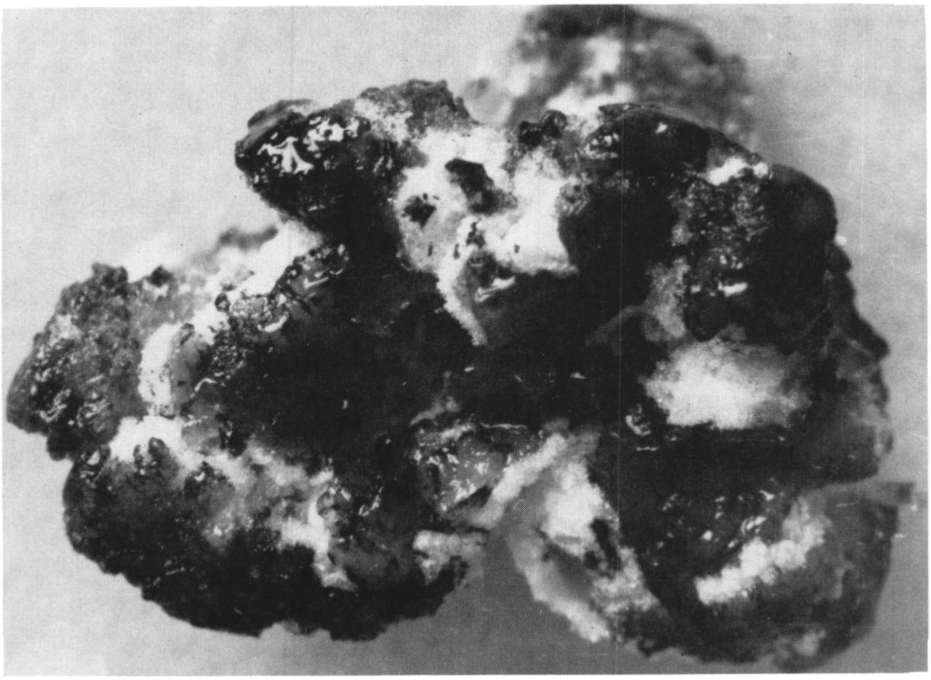

FIGURE 18. Mixed culture of tobacco callus and nitrogen-fixing *Anabaena variabilis*. Dark zones are clusters of cyanobacteria on callus surface.[137] (Magnification × 5.)

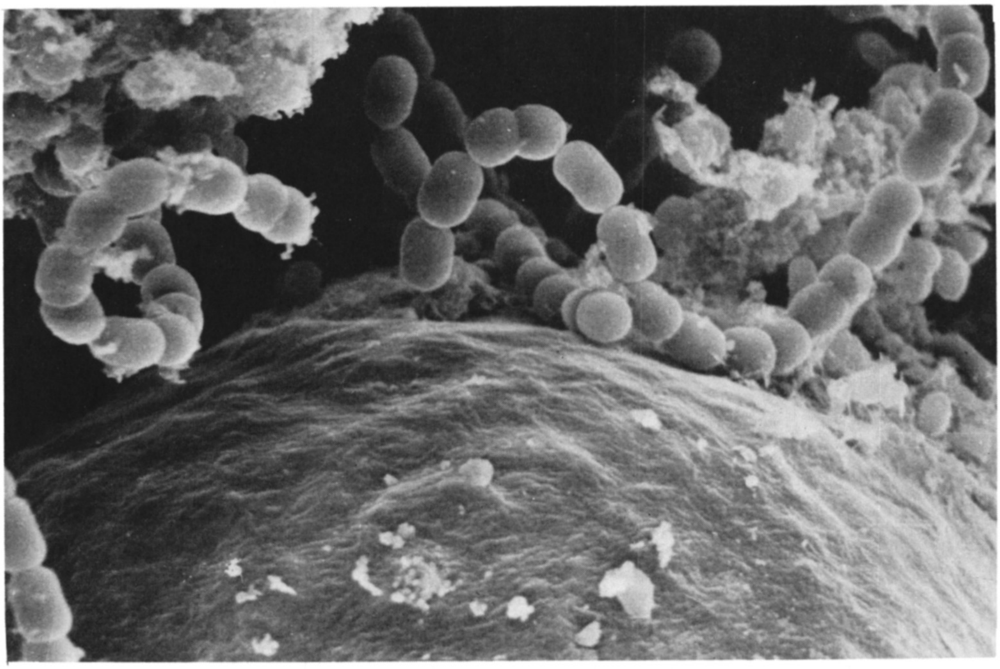

FIGURE 19. Nitrogen-fixing *Anabaena variabilis* on the surface of tobacco callus cell.[137] (SEM; magnification × 2500).

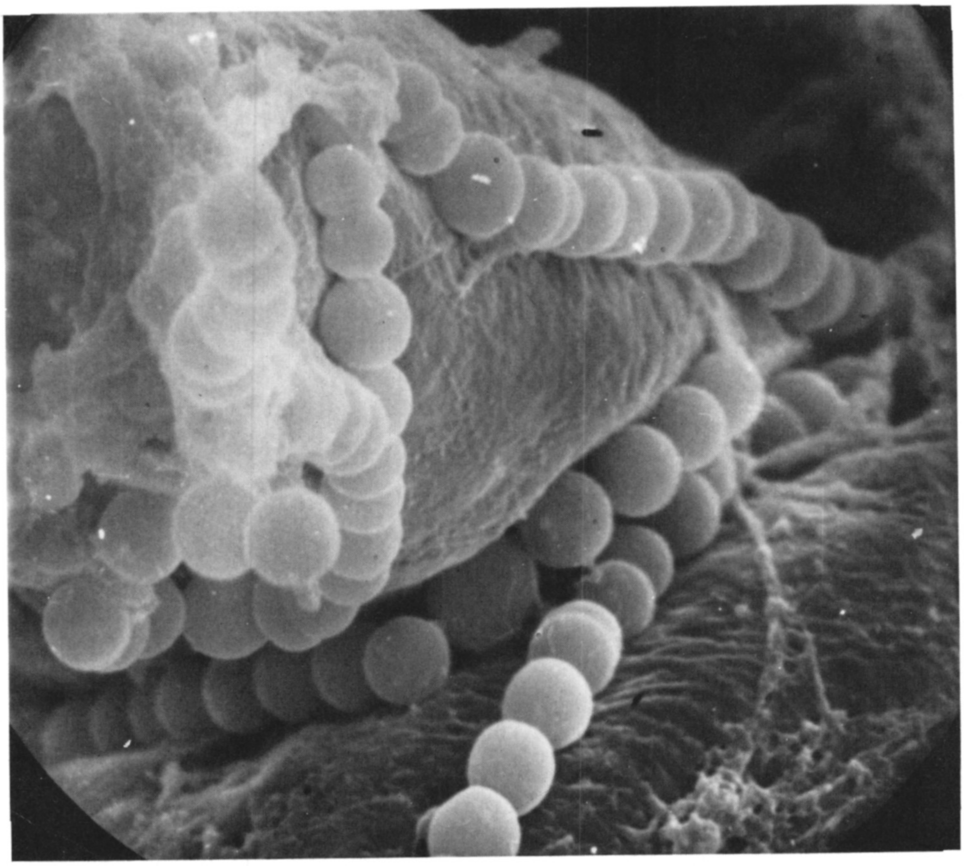

FIGURE 20. *Anabaena variabilis* filaments (nonnitrogen-fixing strain) on the surface and in the depth of tobacco callus.[77] (SEM; magnification × 8250.)

the system obtained showed its stability; no inhibition of the growth of one component by the other was observed.

Introduction of Cyanobacteria into Plants

Mixed cultures of tobacco callus and cyanobacteria were transferred on MS medium for organogenesis with combined nitrogen. Both with nonnitrogen-fixing[144] and nitrogen-fixing[136,137,139] strains of *A. variabilis*, white shoots of tobacco regenerates 2 to 6 cm long, with blue-green zones (Figure 22) had been formed. These zones were formed by large cyanobacterial clusters. This provides evidence of multiplication of the cyanobacteria in shoots obtained via organogenesis. Apparently, such an anatomical feature of tobacco callus as the presence of large intercellular spaces is a determining factor for the penetration of cyanobacteria into the intercellular spaces of the callus tissue and subsequently into the generated shoots. Conversely, the inability of *Azospirillum brasilense* to penetrate deep into sugarcane callus, into the region of meristemoids, is generally thought to be from the absence of intercellular spaces.[87,89]

The ability to form shoots in mixed cultures was maintained during prolonged subculturing (up to 10 or 11 transfers). In mixed culture, as well as in pure callus culture, the ability to regenerate shoots gradually decreased during prolonged subculturing and after the tenth transfer only few cases of regeneration were observed.

The regenerates of the first generation, associated with nitrogen-fixing cyanobacteria, were used for obtaining secondary callus and from this the second generation regener-

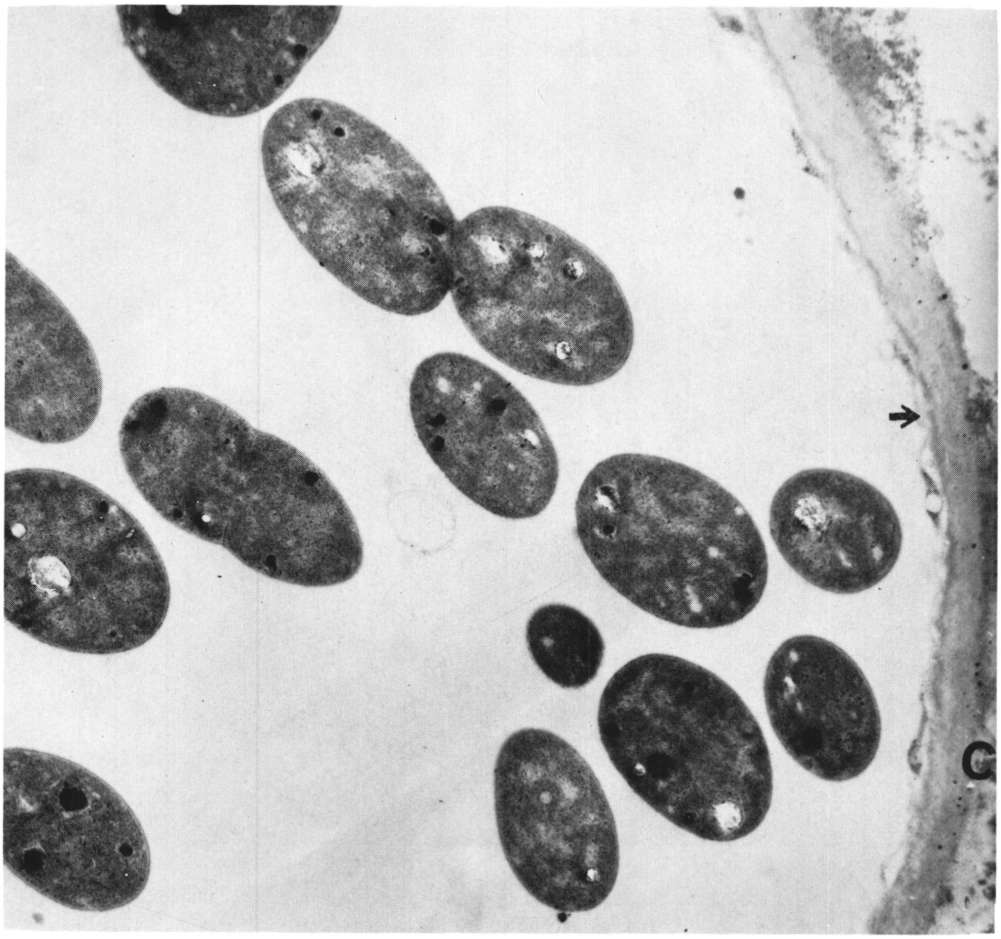

FIGURE 21. Clusters of *Anabaena variabilis* filaments (nonnitrogen-fixing strain) in the central vacuole of a tobacco callus cell; the tobacco cell shows a cell wall (C) and a very narrow layer of cytoplasm (arrow).[77] (TEM; magnification × 9000).

ates.[138,139] These shoots and calli, having passed sequentially through the cycles "plant-callus-shoot-callus-shoot", preserved cyanobacteria on the surface and in the tissues.

Regenerates associated with nitrogen-fixing *Anabaena variabilis* were also obtained by growing alfalfa callus inoculated with cyanobacteria on B5 medium for organogenesis without mineral nitrogen.[130]

To produce associations of tobacco and alfalfa plant with the nitrogen-fixing cyanobacterium *A. variabilis*, different methods for introducing cyanobacteria into plant cuttings were also used.[139]

All attempts to introduce cyanobacteria directly into tobacco shoots propagated by cuttings proved to be a failure. (1) When the cuttings were planted on liquid or agarized MS medium with nitrogen-fixing *A. variabilis*, the cyanobacteria penetrated neither through the stem of the cutting nor through the newly formed root system. On the surface of the roots there was only a slight adsorption of cyanobacteria. (2) Cyanobacteria did not survive on the surface of intact shoots and did not penetrate into the tissues through the sites of cut or the puncture on leaf or stem. (3) Cyanobacteria did not spread along the stem of a shoot, even if they were injected by a fine needle into stems of cuttings. In the latter two cases cyanobacteria did not survive on the place surface and degraded some time later. In one case a plant, in response to wounding with a scalpel on the leaf surface or with a needle at the cutting base, formed callus and cyanobacteria survived there.

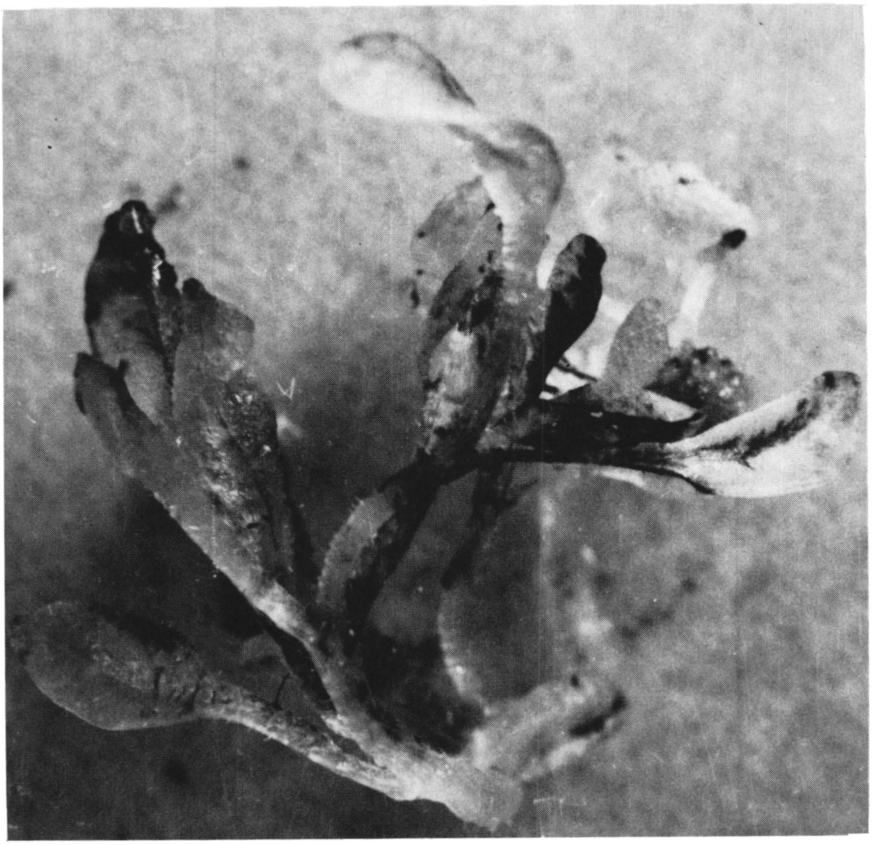

FIGURE 22. Tobacco shoots produced by induction of organogenesis in mixed culture of tobacco callus and *Anabaena variabilis* (nitrogen-fixing strain); dark zones, dots, and lines on the leaves and stems are clusters of cyanobacteria.[139] (Magnification × 5.)

Thus, to establish associations between tobacco shoots and *A. variabilis*, it is necessary to obtain mixed culture as a preliminary stage with subsequent induction of organogenesis. In contrast, inoculation of cuttings or regenerated plants of alfalfa at their base with cyanobacteria, on B5 medium, led to abundant growth of the cyanobacteria in close contact with developing roots.

Localization of Cyanobacteria in Regenerates

In tobacco regenerates, from callus originating from protoplasts, cyanobacteria are localized in the tissue of the stem and leaves.[144] In leaf mesophyll, they are sometimes found inside tobacco cells where there is no cytoplasm; this shows that cyanobacteria retain their intracellular localization during organogenesis.

In tobacco shoots produced from the original callus, cyanobacteria are found both on the surface and in the tissues of the stem and leaves. The folded surface of the stem in this case is filled with chains of *A. variabilis* covered with a finely structured film;[140] the bundles formed by cyanobacterial clusters on the stem surface are oriented from the base to the apex of the shoot. Cross sections of the stem show cyanobacteria in the region of the vascular system. The consistent bunches of long cyanobacterial filaments are localized in the intercellular spaces of the primary cortical stem parenchyma (Figure 23). In the process of organogenesis, cyanobacteria are detected in the xylem vessels (Figure 24) at the base of the regenerating shoots.

Cyanobacteria are found in stomatal pores (Figure 25), under the leaf cuticle (Figure

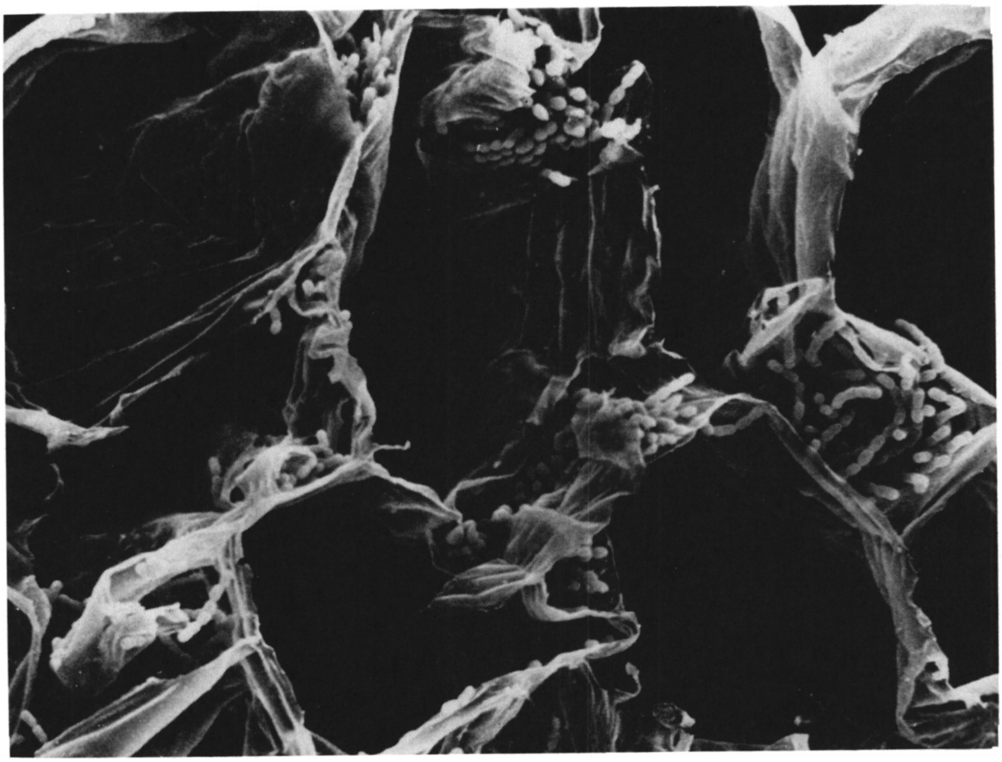

FIGURE 23. Cross section of a tobacco shoot-regenerate stem. The zone of intercellular spaces in the primary cortical parenchyma packed by bunches of *Anabaena variabilis* nitrogen-fixing strain (SEM; magnification × 650).[139]

26), and in specially formed conceptacles covered with a finely structured film (''mantle'') (Figure 27). The film, covering the zones of associative growth of *A. variabilis*, forms artificial conceptacles, inside which the cyanobacteria multiply.

In shoots produced in two cycles, ''plant-callus-shoot-callus-shoot'', the location of the cyanobiont is identical.

In alfalfa regenerates, cyanobacteria are rarely found on the surface or in the tissue of the stem and leaves (Figure 28). In most plant regenerates produced from callus inoculated with cyanobacteria, the cyanobacteria do not remain in the shoots, but exhibit a plentiful growth on the surface and in the tissue of the root (Figures 29 and 30), as in the case of cyanobacteria introduction in the rhizosphere of plants propagated by cuttings. Cyanobacteria attach themselves to the surface rather firmly; they cannot be removed by vigorous shaking of cut roots in liquid medium.

It should be noted that natural symbioses of cyanobacteria with higher plants form special morphological structures which are the localization sites of the cyanobiont. Such structures include distinct cavities at the base of the dorsal lobe of a fern leaf, ageotropic coralloid roots with nodule-like structures in cycads, and stem glands at the leaf bases in *Gunnera* plants.[15,16] In artificial associations, where the evolutionary process has not developed such specialized structures, the location of the symbiont introduced into the plant can also have somewhat similar structures. For example, the intratissue location of cyanobacteria in tobacco shoots coincides with that in a natural association of *Macrozamia communis* with *Nostoc* (which is located in the intercellular spaces of the cortical parenchyma of ageotropic roots).[165] Apart from this, artificial systems also have essential differences in symbiont localization, which are determined by their experimental origin. A cyanobiont in a plant is forced to

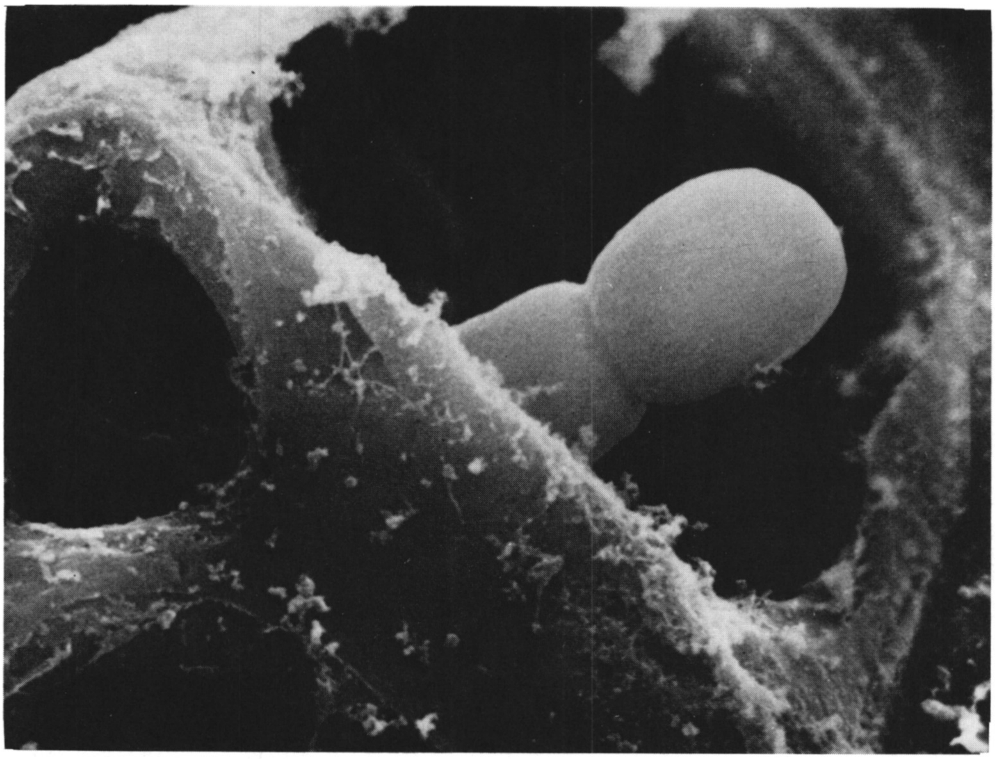

FIGURE 24. *Anabaena variabilis* in a xylem vessel at the base of the forming shoot.[139] (SEM; magnification × 11,000.)

occupy its available parts, "hollows" of the host (elements of vascular system, intercellular spaces, stomatal pores, and central vacuoles of cells), or develops on the plant surface. This can be accompanied by the formation of special conceptacles (cyanobacterial clusters on the surface of a tobacco leaf which are covered with a mucous film). At the same time, the "mantle" found in the artificial associations of tobacco shoots and *A. variabilis* (Figure 27) is identical to the film of the limiting envelope which surrounds the symbiotic cyanobacterium *A. azollae* in the leaf cavity of *Azolla*.[166]

Growth of Callus Tissues and Shoots in Association with Cyanobacteria in the Absence of Combined Nitrogen

Certain experiments provided evidence for the advantages of growing associations of callus tissues and plants with nitrogen-fixing cyanobacteria, compared to nonassociated ones on nitrogen-deficient or nitrogen-free media.

Provided that *Anabaena* and *Plectonema* grew, some variants of mixed culture had a higher proportion of intact carrot cells than the monoculture.[94]

The growth of tobacco callus on nitrogen-free medium ceased in 3 to 4 months. However, the callus associated with nitrogen-fixing cyanobacteria *Anabaena* and *Nostoc* retained its viability and was capable of subculturing for up to 20 months.[142]

At 20% of the normal combined-nitrogen concentration in MS medium, the increase in the biomass of tobacco callus in the mixed culture with *A. variabilis* was 25 to 30% higher than that in the monoculture.[139] Under these conditions and those of long subculturing, a tendency to form meristematic centers in the mixed culture was observed to be 3 d earlier. Under conditions of a long culturing on nitrogen-deficient medium without transfers only the callus tissue areas in contact with the growth zones of cyanobacteria remained viable.

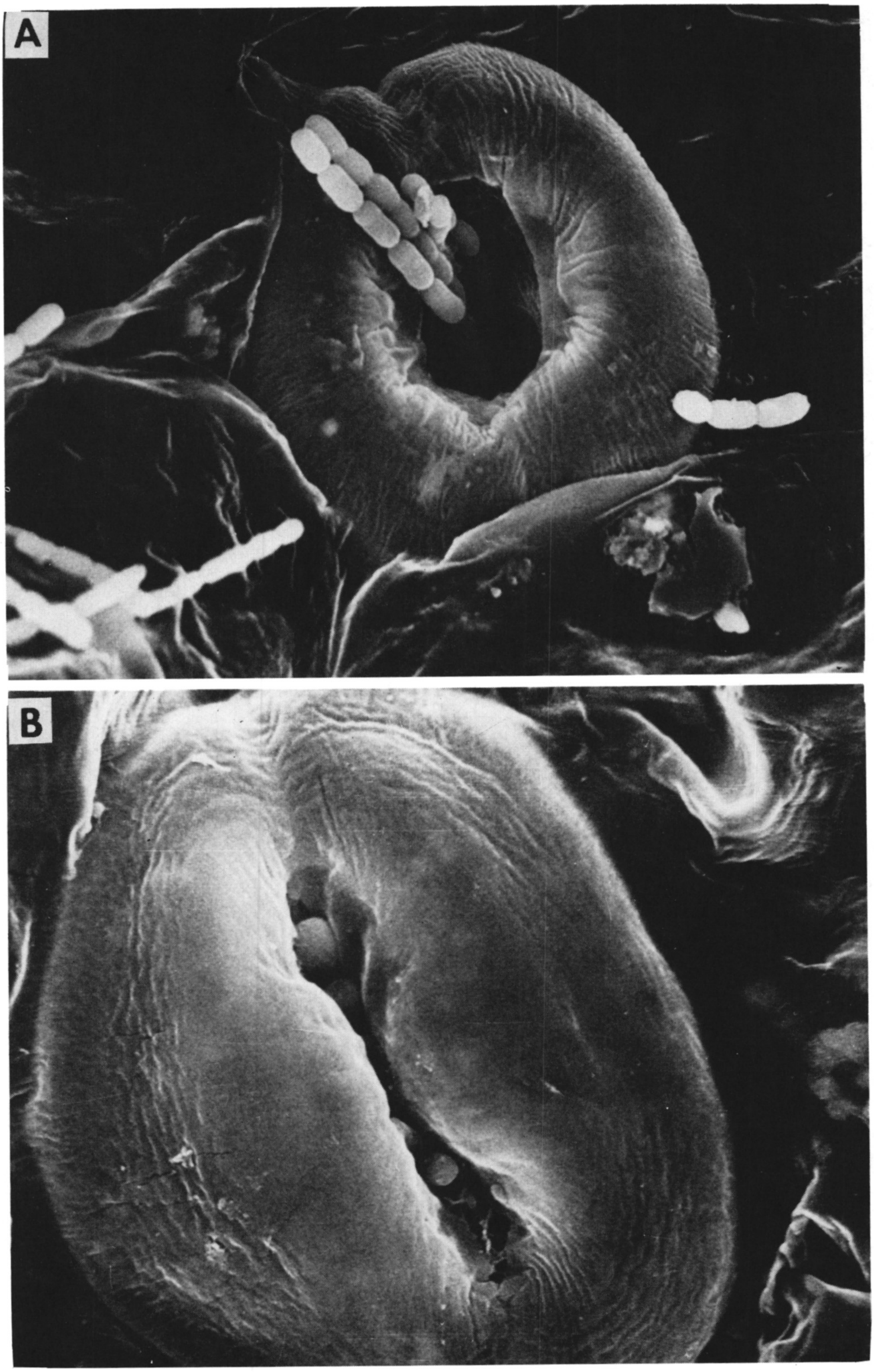

FIGURE 25. *Anabaena variabilis* in the stomatal pore and around the stoma (SEM). The stomatal pore is (A) open[139] and (B) closed.[140] (Magnification [A], × 1900; [B] × 3700.)

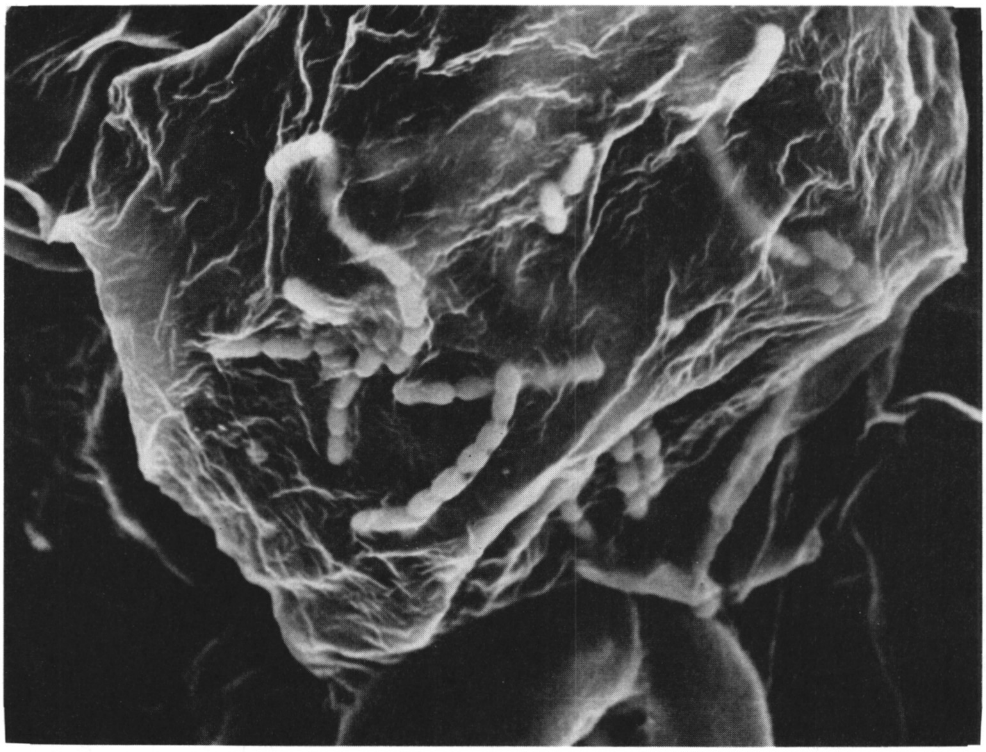

FIGURE 26. *Anabaena variabilis* filaments under leaf cuticle. (SEM; magnification × 1800.)[140]

Finally, cyanobacteria in callus and regenerates caused a delay in the degradation when lower concentrations of combined nitrogen were used. The cells of associated tissues had no osmiophilic pigments (characteristic of nonassociated tissues and aging cells).

With the complete elimination of combined nitrogen from the B5 medium, alfalfa callus in the monoculture did not grow; when cultured together with cyanobacteria, the callus biomass was 2.7 times greater than that in the monoculture (Figure 31). In the coculture, practically all the alfalfa cells (94%) in 10 d of growth remained viable, whereas in the monoculture only 54% remained viable. Unlike the previously used mixed cultures, this experiment employed a coculture in which callus and cyanobacteria were grown, spaced from each other, on agarized medium, i.e., without partner cells being in contact. The associations of alfalfa plants and *A. variabilis* were found to grow on nitrogen-free B5 medium (they were observed for 5 months). Noninoculated plants under the same conditions showed signs of degradation within 1 month, after 2 months their leaves discolored and fell, and shortly thereafter the plants died. The inoculated plants continued to grow in sandy culture without combined nitrogen. Cyanobacteria in this case were localized on the surface of the roots, predominantly in the root hair zone, in contact with root hairs, and spread to those areas of the roots which had formed during their growth after the plants had been transferred to the sand. Moreover, the inoculated and control (noninoculated with cyanobacteria) plants were twice as tall after 10 d of growth in the sandy culture. The plants associated with cyanobacteria also grew in sterile soil, and after 4 months of growth the cyanobacteria intensively developed both in direct contact with the roots and in the rhizosphere.

Thus, the results of these experiments have shown for the first time that nitrogen-fixing bacteria in artificial associations can support the growth of callus, and a whole plant, on medium or sandy culture without combined nitrogen. Moreover, by using the coculture it

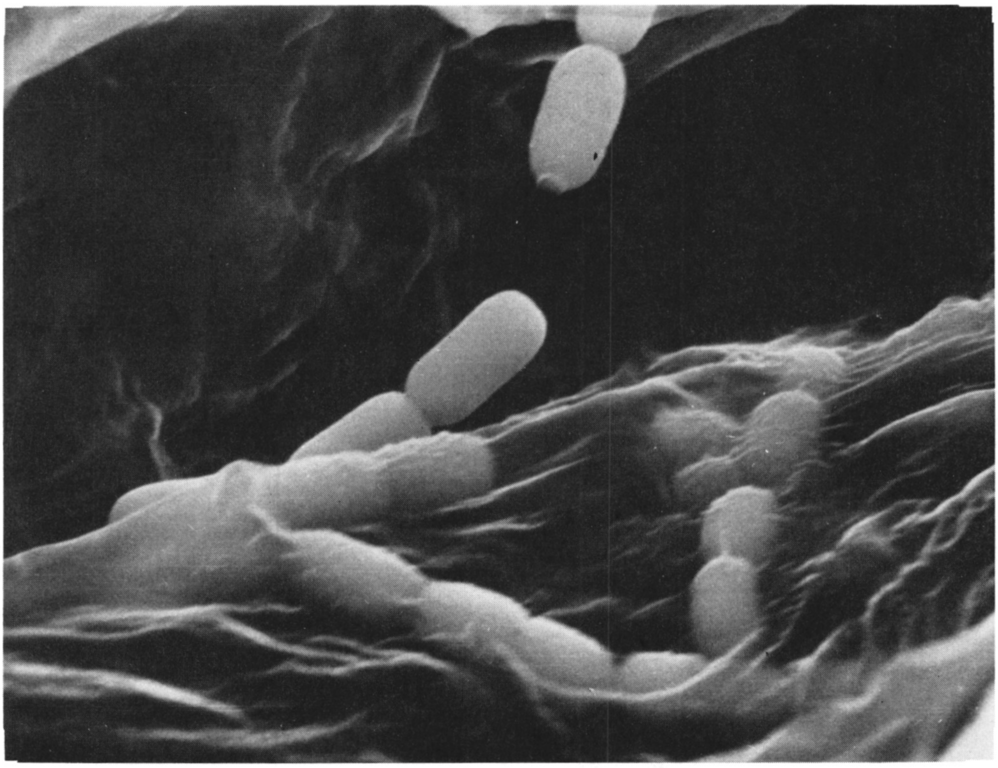

FIGURE 27. *Anabaena variabilis* on the leaf surface, under mucous film ("mantle"), and on its surface.[140] (SEM; magnification × 6300.)

was possible to demonstrate that fixed N, produced by cyanobacterial nitrogen fixation, is excreted into the medium and made available to the callus by diffusion through the agar.

Nitrogenase Activity

On MS and B5 media, used for culturing tobacco and alfalfa, respectively, *A. variabilis* did not grow in monoculture, had a low level of heterocysts and ARA (Figure 31), and eventually died.

In associations with tobacco callus and regenerates,[137,139] with alfalfa suspension and callus cultures and plants,[130] and in a coculture with alfalfa callus, cyanobacteria multiply rapidly, form heterocysts, and exhibit ARA. The formation of heterocysts, which are responsible in the given cyanobacterium for the nitrogen fixation, in mixed cultures and regenerates was observed both on full medium (Figures 32 and 33) and on nitrogen-deficient medium (Table 7; Figures 31, 34, and 35). The formation of heterocysts and recording of ARA provide evidence for nitrogen fixation by cyanobacteria. When tobacco callus was grown on nitrogen-free medium with cyanobacteria, the formation of ethylene was ten times as high as the endogenous formation of ethylene in the calli grown under the same conditions without cyanobacteria as well as in the calli with cyanobacteria where no acetylene was added.[141]

ARA and the frequency of heterocysts, in associations of *A. variabilis* with regenerated tobacco shoots, increased with a reduction in the concentration of combined nitrogen in the medium (Table 7). This activity was higher in the regenerated shoots than in pure cultures grown under the same conditions. These ARA values are consistent with those for cyanobacteria isolated from natural associations with higher plants.[153,168] The callus and tissues of regenerated shoots containing no cyanobacteria lacked such activity, thus implying that cyanobacteria are responsible for the manifestation of this activity in associated tissues.

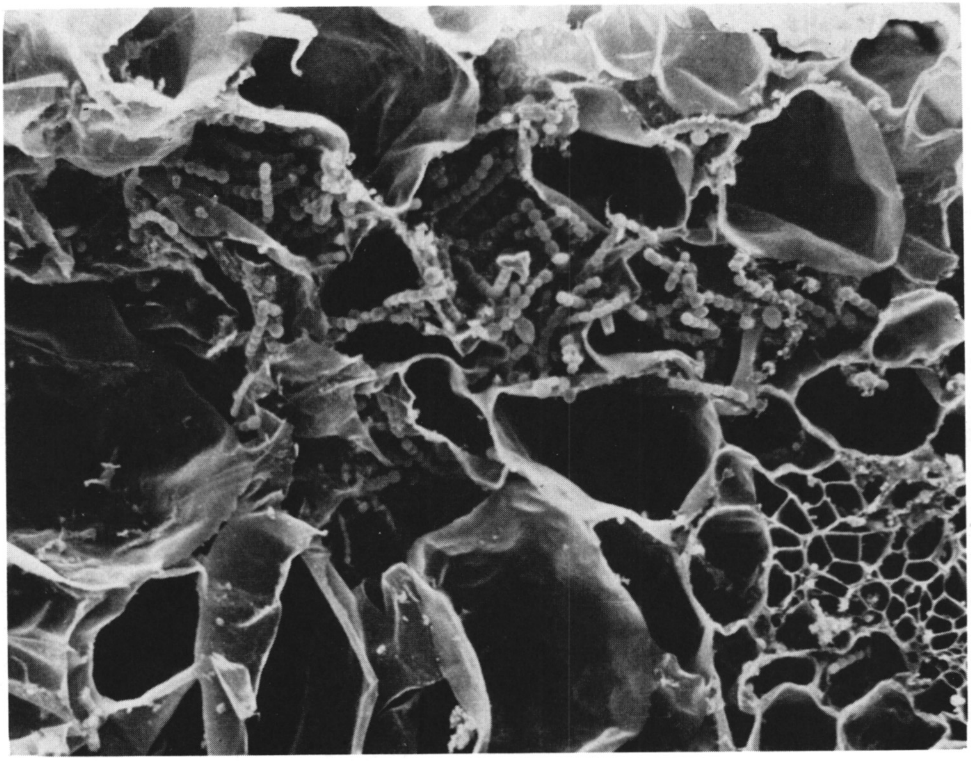

FIGURE 28. Cross section of a leaf from a regenerated alfalfa plant; *Anabaena variabilis* clusters in intercellular spaces of leaf mesophyll. (SEM; multiplication × 400.)

Compared to the cyanobacteria in pure culture, the cyanobacteria grown in association with alfalfa cells and regenerates have a higher heterocyst frequency (Figure 35). ARA values in this case are comparable to those known from the literature for cyanobacteria in natural symbioses.[16] Special experiments with a coculture of alfalfa tissue and *A. variabilis* showed that the growth, ARA, and the formation of heterocysts in cyanobacteria (Figure 31) were obviously stimulated by alfalfa cell metabolites diffusing through agar.

Thus, plant cells and tissues, in addition to stimulating nitrogenase in *Rhizobium* and *Azospirillum*, cause a similar effect in the nitrogen-fixing cyanobacterium *A. variabilis*. As shown earlier with *Rhizobium* and *Azospirillum*, experimental data point to the existence of metabolites, excreted by plant cells and tissues, which are necessary for growth and nitrogen fixation of cyanobacteria on MS and B5 media.

It is noteworthy that higher plants in natural associations also stimulate the formation of heterocysts and nitrogenase in the cyanobiont.[16] However, in artificial systems the frequency of heterocysts did not reach maximum values known for natural symbioses of cyanobacteria and higher plants. The notable property of cyanobacteria in artificial associations is its plentiful growth which, in natural associations with plants is inhibited.

Thus, in artificial associations with higher plant cells, tissues and regenerates cyanobacteria seem to fix nitrogen. This may account for the advantages found previously for plant tissues grown in association with nitrogen-fixing cyanobacteria on nitrogen-deficient media. The plant tissues, in turn, exert a stimulating effect on the formation of heterocysts and ARA in cyanobacteria. It should be noted that, as far as we know, there is only one report of finding a cyanobacterium (*N. punctiforme*) in the nodules of a leguminous plant *Trifolium alexandrinum*.[169]

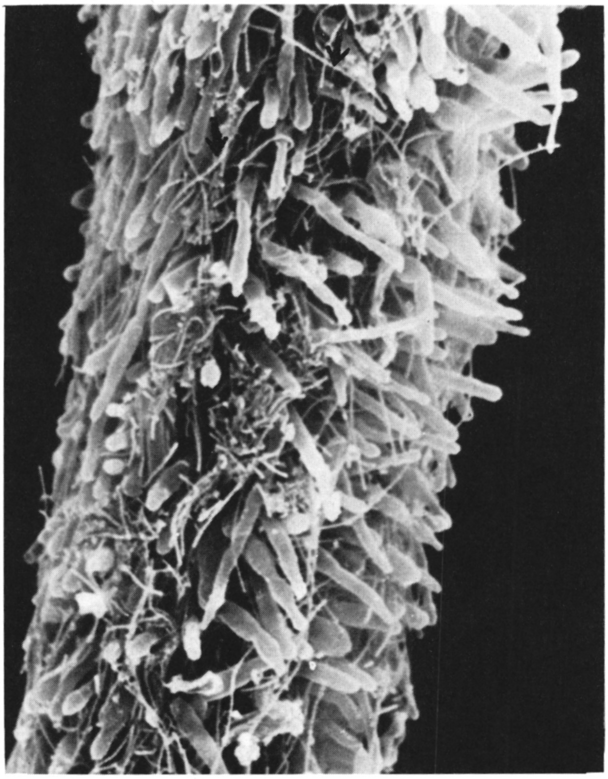

FIGURE 29. *Anabaena variabilis* in the root hair zone of a regenerated alfalfa plant. (SEM; magnification × 80.)

Uptake of Plant Metabolites by Cyanobacteria

In natural associations with higher plants, cyanobacteria provide the plant with combined nitrogen and receive carbon compounds from the plant.[16] One of the problems in the practical use of artificial associations, as noted at the beginning of this chapter, is the supply of organic compounds to the nitrogen-fixing symbiont without damaging the host plant. In association with alfalfa plants, cyanobacteria grow heterotrophically in sandy culture and soil; apparently, this is ensured by root excretions of alfalfa.

Special tests have been carried out to ascertain the possibility of transferring carbon compounds from plants to cyanobacteria in an artificial association.[170] Tobacco shoots were initially fed with ^{14}C-labeled glucose. Cyanobacterial cells were then applied to the leaf surfaces of such ^{14}C-labeled plants and were exposed, together, for a certain time. It should be noted that in the one case cyanobacteria were taken directly from pure culture (nonadapted) and in the other case they were isolated from callus with which they had been jointly grown in subculture for 1.5 years (adapted). The regenerated plants transmitted carbon-labeled compounds to cyanobacteria both in the light and in the dark, as evidenced by ^{14}C movement from the plant to the cyanobacterium (Figure 36).

It is interesting to note at this point that the adapted cyanobacteria receive 2.5 times as much carbon-labeled compounds from the shoots, both in the light and dark, as nonadapted cyanobacteria. The difference in the incorporation of labeled organic matter into adapted and nonadapted cyanobacteria points to the fact that under conditions of association with tobacco callus culture, *A. variabilis* passes through a complex adaptive period accompanied by metabolic rearrangements. Obviously, this is one of the reasons that the associations of tobacco shoots and cyanobacteria can be established only through the use of a mixed culture, which seems to be the adaptive stage of the cyanobacteria to new conditions of their existence.

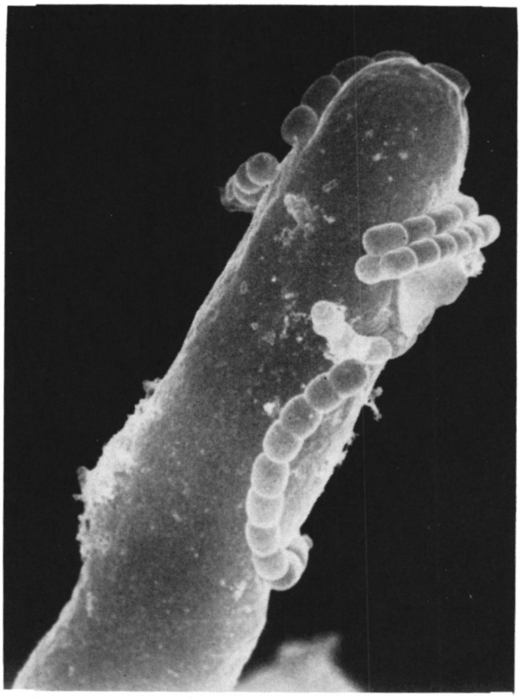

FIGURE 30. *Anabaena variabilis* filaments in contact with a root hair of an alfalfa regenerate. (SEM; magnification 2300.)

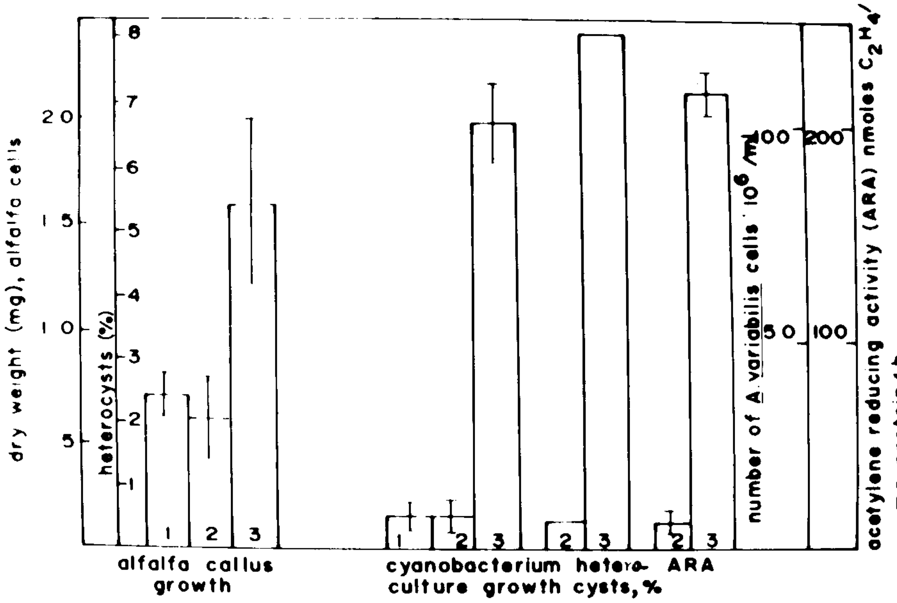

FIGURE 31. Growth of alfalfa callus, and growth and ARA of *Anabaena variabilis*, in monocultures and cocultures on nitrogen-free B5 medium. (1) Initial inoculum; (2) monoculture; (3) coculture. (Growth period 14 d.)

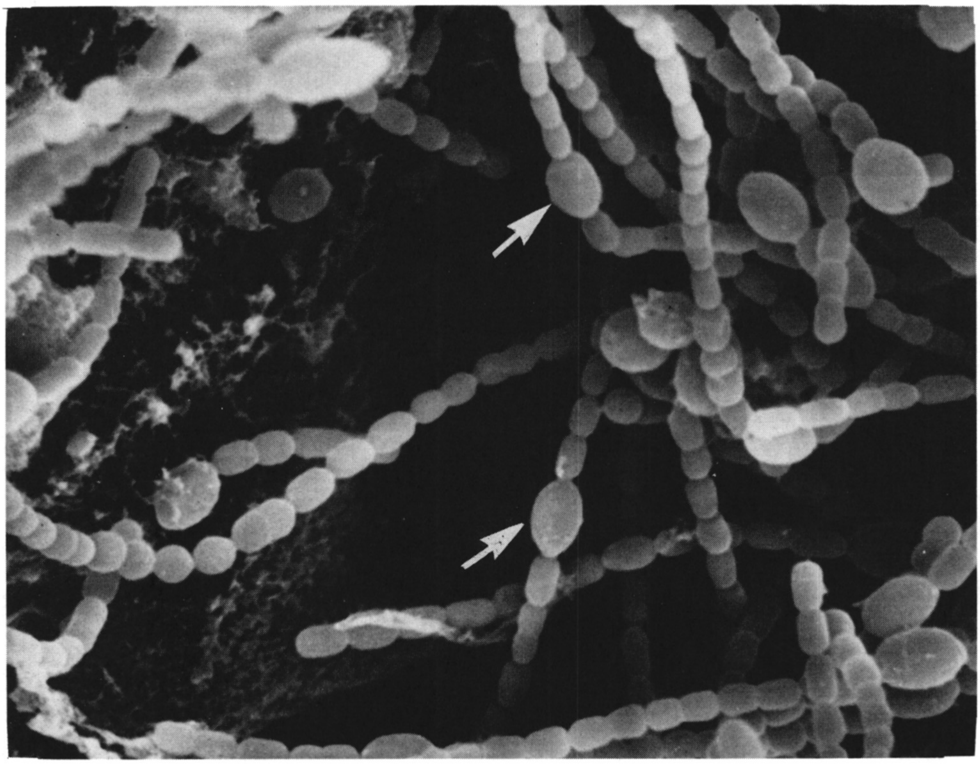

FIGURE 32. Cyanobacterial clusters on the surface of a tobacco shoot leaf (heterocysts indicated by arrows). Long culturing on MS medium with combined nitrogen for 3 months.[139] (Magnification × 1800.)

Thus, the above experiments have demonstrated the establishment of artificial associations of cultured tissues and regenerated plants with cyanobacteria. It was possible to produce associations of cyanobacteria with callus and shoots obtained from isolated protoplasts. The cell systems obtained have been shown to possess a high stability during their culturing and to preserve associative interactions during the transfers.

CONCLUSION

Analysis of work on designing artificial associations of various types has revealed that these experiments involved a wide variety of plant objects and microorganisms. Cyanobacteria seem to have been the most widely used microorganisms (except for *Rhizobium*) for producing both endo- and exosymbiotic associations. No intracellular-type associations have been obtained by introduction of either cyanobacteria or other microorganisms into isolated plant protoplasts (Table 1). The failures are most likely due to the inadequacy of the introduction technique or to an insufficiently wide set of the tested partner combinations.

Experiments with mixed cultures have demonstrated improvement of the growth and biosynthetic parameters of the cells (tissues) cultured in association with various groups of microorganisms (Table 2), including cyanobacteria (Table 3). Only in mixed culture, plants associated with cyanobacteria have been regenerated. Furthermore, nutrient exchange between partners in such symbioses has also been demonstrated to occur.

The accumulated experimental data form a sound basis for selection of suitable partner combinations for joint culturing and producing new cell systems with predetermined properties. At this point, the results of introducing cyanobacteria into populations of cultured

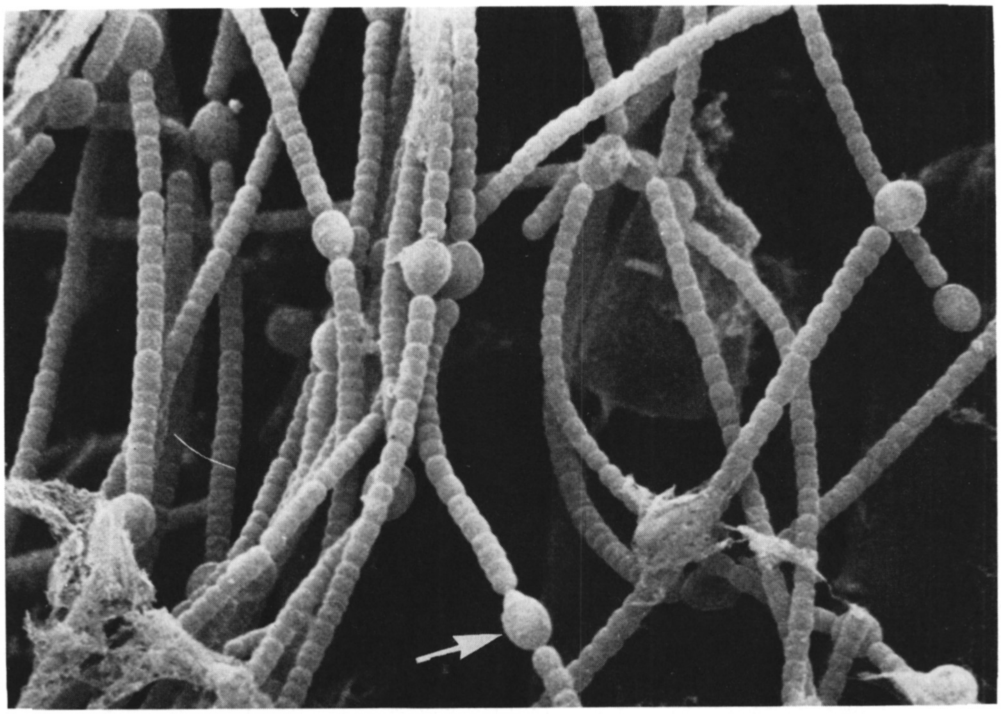

FIGURE 33. *Anabaena variabilis* heterocysts (arrows) on the surface of alfalfa root; grown on B5 medium with combined nitrogen. (SEM; magnification × 1700.)

TABLE 7
Acetylene Reduction Activity (ARA) and Heterocyst Frequency of *Anabaena variabilis* in Pure Culture and in Tobacco Regenerates[139]

Sample	**Medium**	**Content of combined nitrogen (% of normal)**	**Acetylene reduction (mmol $C_2H_4 \cdot 10^{-3} \cdot$ (mg Chl)$^{-1} \cdot h^{-1}$)**	**Heterocyst frequency (%)**
Regenerated shoot tissue with cyanobacteria	MS	100	0.39 ± 0.028	(1) 0.3 ± 0.039 (2) 0.4 ± 0.038
	MS	20	6.0 ± 1.01	(1) 0.3 ± 0.039 (2) 7.0 ± 0.71
	MS	0	9.7 ± 0.8	(1) 0.3 ± 0.022 (2) 7.3 ± 0.83
Regenerated shoot tissue free of cyanobacteria	MS	100, 20, 0	0	—
Pure culture of *A. variabilis*	MS	20	3.4 ± 0.53	(1) 7.0 ± 0.38 (2) 7.0 ± 0.38
	MS	0	4.2 ± 0.21	(1) 7.0 ± 0.52 (2) 7.2 ± 0.50
	AA	0	8.3 ± 0.31	(1) 7.0 ± 0.38 (2) 7.2 ± 0.48

Note: MS = Murashige and Skoog; AA = Allen and Arnon. (1) = before transfer; (2) = after transfer from MS organogenetic medium with 40% nitrogen (compared with full combined-nitrogen content).

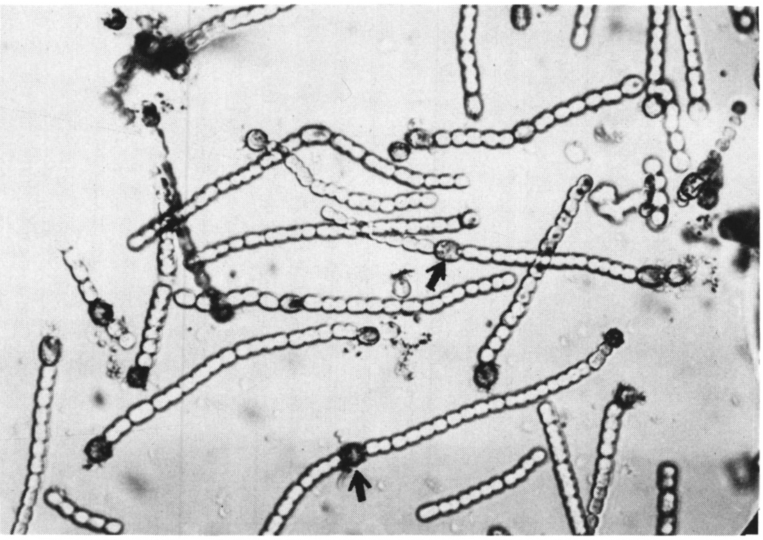

FIGURE 34. Identification of heterocysts (arrow) in *Anabaena variabilis* filaments by their ability to reduce 2,3,5-triphenyl tetrazolium chloride to formazan;[167] mixed suspension culture with alfalfa cells. (Light microscopy; magnification × 450.)[167]

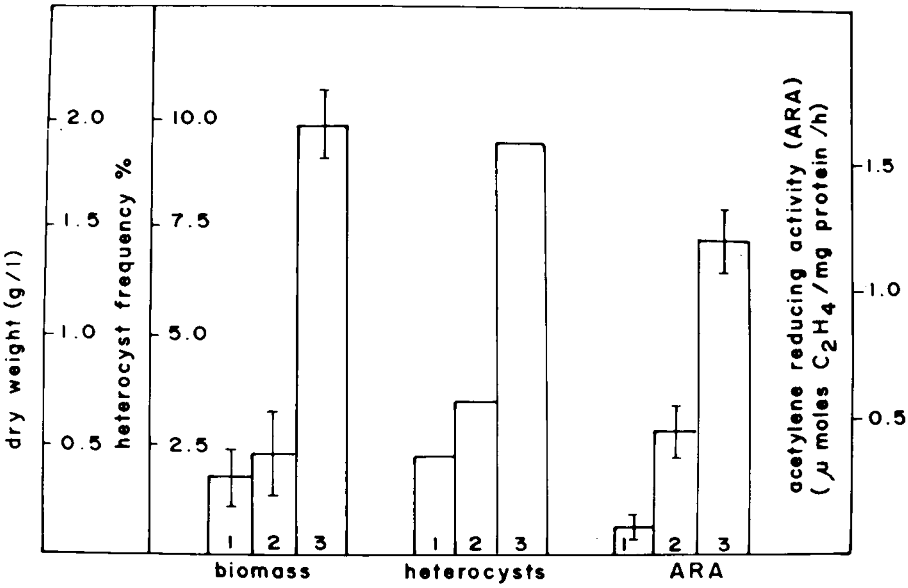

FIGURE 35. Stimulating effect of alfalfa cells on the growth, heterocyst formation, and ARA of *Anabaena variabilis*.[130] Growth variants of *A. variabilis* (in suspension cultures); (1) in monocultures on B5 medium, (2) on AA medium, and (3) in mixed culture with alfalfa cells on B5 medium. Nitrogen-free media were used.

cells could be of great assistance in selecting compatible combinations of partners (Table 3). However, it should be borne in mind that the conditions of intracellular existence of a cyanobiont may vary greatly from those of their growth in mixed cultures or in plants (outside the cells).

In established associations, cyanobacteria can function as a source of substrates for the growth as well as for the biosynthesis of species-specific products by plant cells. Plant cells

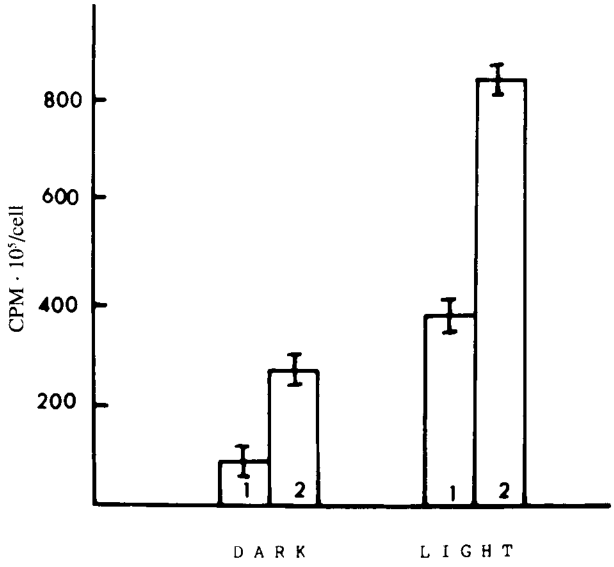

FIGURE 36. Uptake of ^{14}C-compounds by *Anabaena variabilis* cells. (1) Unadapted; (2) adapted. Radioactivity of cyanobacteria was assessed after preliminary contact with tobacco shoots labeled with ^{14}C-glucose. Radioactivity of cyanobacteria washed off from unlabeled regenerates was (0.02 ± 0.015) · 10^{-5} cpm per cell.

have been shown to utilize photosynthetic products of cyanobacteria.[121] In all other cases of using phototrophic microorganisms, including numerous experiments with cyanobacteria, no objectives for applying their photosynthetic potential in artificial associations have been set. The growth of plant cells can also be supported by uptake of cyanobacterial cell lysates. In the presence of cyanobacteria, the biosynthetic activity of plant cells is preserved or even increased.[122,132,133] Rough estimates have revealed the efficiency of such cyanobacterial systems in obtaining valuable natural products. All this is of much interest in terms of prospects of using the method of mixed culturing, based on plant cells, in biotechnology.

In mixed cultures and cocultures with plant cells, and in associations with plants/regenerates, cyanobacteria can function as a nitrogen-fixing component of the systems. Nitrogen-fixing cyanobacteria have been shown to be able to support the growth of plant tissues and plants. Cells and tissues of various plant species stimulate or induce nitrogenase activity in all the studied nitrogen-fixing bacteria (Table 2). A similar effect has been demonstrated for alfalfa cells (tissues) on ARA and formation of heterocysts in *A. variabilis* (Figure 35). Apart from this, the ARA of cyanobacteria in regenerated tobacco shoots is also higher than that of cyanobacteria grown in pure culture under the same conditions (Table 7). Thus, the influence of plant cells and tissues on nitrogen fixation in cyanobacteria is similar to their effect on this process in other microorganisms. This effect is also similar to the influence of higher plants on the nitrogen-fixing symbionts in natural associations.

Previous attempts at establishing associations of regenerated plants with bacteria, *Azotobacter vinelandii*[38] and *Azospirillum brasilense*,[87,89] have been unsuccessful. In contrast, it has been possible to do so with nitrogen-fixing cyanobacteria. At this point it should be noted that tobacco plants cannot be infected with cyanobacteria without a preliminary stage of mixed culturing. It is possible that the failure in directly infecting differentiated tissues (cuttings and roots) is due to the presence of a mechanism rejecting cyanobacteria in mature plants. Plants are known to possess protective properties and resistance to infection, whereas in the callus tissues this barrier is inefficient in a number of cases.[171]

In the formation of associations with higher plants in nature, much importance is attached to the specific properties of the surface structures in the partners.[16] Similarly, as discussed earlier, surface structures of plant cells and cyanobacteria are important in establishing artificial associations.[8]

Higher plant tissues have been shown to possess a specific antigenic composition depending on the extent of their differentiation.[172] The callus and regenerates are characterized by the antigen common for them, but they lack the root antigens.[173] The antigenic structures may undergo some changes during the existence of *Anabaena variabilis* in callus. Indeed, symbiotic cyanobacteria are known to differ from those cultured in isolation, regarding their antigenic characters.[174] Finally, it may be assumed that cyanobacterial cells require adaptive rearrangement in the mixed culture with callus to prepare them for the existence in plant-regenerates. A better susceptibility of alfalfa plants to cyanobacterial infection possibly results from the fact that they are adapted to symbiotic existence with *Rhizobium*.

The experimental models of artificial associations discussed above can be useful in studies on the processes of rejection and susceptibility to infection among partners. In addition, these systems may be used to solve such problems as: (1) studies, at cellular level, on the mechanisms underlying the influence of a plant host on symbiotic cyanobacteria; (2) search for factors which control nitrogen fixation in cyanobacteria; and (3) experimental establishment of new efficient nitrogen-fixing associations.

Of special interest is the coculturing of isolated protoplasts with adsorbed cyanobacteria, leading to the formation of associated callus[77] and associations of regenerated shoots with cyanobacteria.[144] In callus culture and shoots, cyanobacteria are found both in the intercellular spaces and inside the cells. This forms the basis for referring to the given systems, in terms of localization of the introduced cyanobacteria, as the mixed (intermediate) type associations. To obtain callus and plants with entirely intracellular localization of the symbiont, it seems necessary to carry out special tests on the selection of intracellular systems, with cyanobacteria being introduced into protoplasts. It is difficult to predict now (1) which system, intracellular or intercellular, will be superior for the formation and survival of associations and (2) whether the initial intracellular localization of the symbiont will be preserved as such during regeneration.

Localization of cyanobacteria and relations of partners in artificial associations have certain similarities with natural symbioses, including: (1) the stimulating effect of plant cells and tissues on the formation of heterocysts and ARA in cyanobacteria; (2) support of growth of plant tissues and plants by cyanobacteria by supplying fixed nitrogen; (3) transfer of carbon compounds from plant tissues to nitrogen-fixing cyanobacteria; (4) supply of cyanobacterial photosynthetic products to the heterotrophic partner of the association; (5) location of the cyanobiont is, as a rule, outside the cells of the partner and rarely intracellular; (6) formation of mucuses at location sites of cyanobacteria.

The considerable differences between artificial and natural associations of cyanobacteria are as follows: (1) in natural associations cyanobiont growth is inhibited or reduced in a plant host, whereas in an artificial association it is stimulated by metabolites from the plant partner; (2) formation of heterocysts in the cyanobacteria is not as high as that in the cyanobacteria of natural symbioses; (3) due to lack of specialized structures characteristic of cyanobiont location in natural symbioses, the cyanobiont in artificial associations finds (or specially forms) its habitats in populations of plant cell (tissue) cultures or in plants.

Returning to the problems that were posed 10 years ago in experiments on establishing artificial associations,[39] it is safe to say that the experiments carried out over this period have provided evidence for the possibility of solving these problems by using model systems.

REFERENCES

1. **Rodgers, G. A. and Stewart, W. D. P.,** The cyanophyte-hepatic symbiosis. I. Morphology and physiology, *New Phytol.*, 78, 441, 1977.
2. **Bonnet, H. T. and Silvester, W. B.,** Specificity in the *Gunnera-Nostoc* endosymbiosis, *New Phytol.*, 89, 121, 1981.
3. **Meeks, J. C., Enderlin, C. S., Chapman, J. S., and Joseph, C. M.,** Nitrogen assimilation in symbiotic *Nostoc* reconstituted in pure culture with the hornwort *Anthoceros*, in *Abstr. 4th Int. Symp. Photosynthetic Prokaryotes*, Bombannes, France, 1982, E 3.
4. **Enderlin, C. S. and Meeks, J. C.,** Pure culture and reconstitution of the *Anthoceros-Nostoc* symbiotic association, *Planta*, 158, 157, 1983.
5. **Fowke, L. C. and Gamborg, O. L.,** Applications of protoplasts to the study of plant cells, *Int. Rev. Cytol.*, 68, 9, 1980.
6. **Giles, K. L. and Vasil, I. K.,** Nitrogen fixation and plant tissue culture, *Int. Rev. Cytol.*, Suppl. 11B, 81, 1980.
7. **Gamborg, O. L. and Bottino, P. J.,** Protoplasts in genetic modifications of plants, in *Advances in Biochemical Engineering*, Vol. 19, Fiechter, A., Ed., Springer-Verlag, Berlin, 1981, 239.
8. **Gusev, M. V., Butenko, R. G., and Korzhenevskaya, T. G.,** Cyanobacteria in association with cultivated cells of higher plants, in *Soviet Scientific Reviews: Physicochemical Biology Reviews*, Vol. 4, Skulachev, V. P., Ed., Harwood Academic Publishers, New York, 1984, 1.
9. **Reisser, W.,** Endosymbiotic cyanobacteria and cyanellae, in *Encyclopedia of Plant Physiology*, New Series, Vol. 17, Linskens, H. and Heslop-Harrison, J., Eds., Springer-Verlag, Berlin, 1984, 91.
10. **Kovacheva, N., Selenska, S., and Atanasov, A.,** Artificial nitrogen fixing systems between cyanobacteria and higher plants, *Biotechnol. Bioind.*, 2, 25, 1986 (in Bulgarian).
11. **Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.,** Associations of cyanobacteria with cultivated cells and tissues of higher plants, in *Plant Cell Culture and Biotechnology*, Butenko, R. G., Ed., Nauka, Moscow, 1986, 225.
12. **Margulis, L.,** *Symbioses in Cell Evolution*, W. H. Freeman, San Francisco, 1981.
13. **Gusev, M. V. and Gochlerner, G. B.,** *Free Oxygen and Evolution of the Cell*, Moscow State University Press, Moscow, 1980, chap. 4 (in Russian).
14. **Stewart, W. D. P.,** A botanical ramble among the blue-green algae, *Br. Phycol. J.*, 12, 89, 1977.
15. **Peters, G. A.,** Blue-green algae and algal associations, *BioScience*, 28, 580, 1978.
16. **Stewart, W. D. P., Rowell, P., and Rai, A. N.,** Cyanobacteria-eukaryotic plant symbioses, *Ann. Microbiol. (Inst. Pasteur)*, 134, 205, 1983.
17. **Torrey, J. G.,** *In vitro* methods in the study of symbiosis, in *Proc. 4th Int. Congr. Plant Tissue and Cell Culture*, Thorpe, T. A., Ed., University of Calgary Press, Calgary, 1980, 373.
18. **Yurkova, G. N. and Levenko, B. N.,** Artificial symbiotic nitrogen-fixing systems, *Mol. Biol. (Kiev)*, 19, 63, 1978, (in Russian).
19. **King, P. J. and Street, H. E.,** Growth patterns in cell cultures, in *Botanical Monographs*, Vol. 11, 2nd ed., Street, H. E., Ed., Blackwell Scientific, Oxford, 1977, 307.
20. **Fuller, K. W.,** Chemicals from plant cell cultures — some biochemical and physiological pointers, *Chem. Ind. (London)*, 23, 825, 1984.
21. **Butenko, R. G.,** Cell technologies for production of economically valuable substances of plant origin, in *Plant Cell Culture and Biotechnology*, Butenko, R. G., Ed., Nauka, Moscow, 1986, 3 (in Russian).
22. **Fujita, Y., Tabata, M., Nishi, A., and Yamada, Y.,** New medium and production of secondary compounds with the two-staged culture method, in *Proc. 5th Int. Congr. Plant Tissue and Cell Culture*, Fujiwara, A., Ed., ABC Photoprinting, Tokyo, 1982, 399.
23. **Hashimoto, T., Azechi, S., Sujita, S., and Suzuki, K.,** Large scale production of tobacco cells by continuous cultivation, in *Proc. 5th Int. Congr. Plant Tissue and Cell Culture*, Fujiwara, A., Ed., ABC Photoprinting, Tokyo, 1982, 403.
24. **Butenko, R. G.,** *Potential Uses of Plant Cultured Cells in Biotechnology*, Baev, A. A., Ed., Nauka, Moscow, 1984, 239 (in Russian).
25. **Yeomann, M. M. and Macleod, A. J.,** Tissue (callus) cultures-techniques, in *Botanical Monographs*, Vol. 11, 2nd ed., Street, H. E., Ed., Blackwell Scientific, Oxford, 1977, 31.
26. **Butenko, R. G.,** Physiological study of cell cultures: current state and prospects, *Fiziol. Rast.*, 25, 1009, 1978 (in Russian).
27. **Egorov, N. S., Landau, N. S., and Gesheva, V. I.,** Study of the formation of fibrinolytic exoproteases in the associative cultures of actinomycetes, *Appl. Biochem. Microbiol.*, 18, 383, 1982 (in Russian).
28. **Vorobiev, A. A. and Korovkin, V. I.,** Prospects for biotechnological developments and patent regulations as to the patent assignment of the title "invention" to innovations in this field, *Zh. Mikrobiol. Epidemiol. Immunobiol.*, 10, 13, 1983 (in Russian).

29. **Gutschick, V. P.,** Energy flows in the nitrogen cycle, especially in fixation, in *Nitrogen Fixation,* Vol. 1, Newton, W. E. and Orme-Johnson, W. H., Eds., University Park Press, Baltimore, 1980, 17.
30. **Preussmann, R., Eisenbrand, G., and Spiegelhalder, B.,** Occurrence and formation of N-nitroso compounds in the environment and *in vivo,* in *Environ. Carcinogenesis,* Elsevier, Amsterdam, 1979, 51.
31. **Lebedev, E. M.,** Possible ecological consequences of using superfluous quantities of nitrogen fertilizers, in *Mineral and Biological Nitrogen in Agronomy in the USSR,* Nauka, Moscow, 1985, 41 (in Russian).
32. **Pühler, A.,** Die Molekularbiologie der Stickstoffixierung bei *Klebsiella pneumoniae, Biologie in Unserer Zeit,* 9, 14, 1979.
33. **Peters, G. A. and Calvert, H. E.,** The *Azolla-Anabaena azollae* symbiosis, in *Algal Symbiosis,* Goff, L. J., Ed., Cambridge University Press, Cambridge, 1983, 103.
34. **Vose, P. B.,** Developments in nonlegume N_2-fixing systems, *Can. J. Microbiol.,* 29, 837, 1983.
35. **Schell, J. and Van Montagu, M.,** The Ti-plasmid of *Agrobacterium tumefaciens,* a natural vector for the introduction of *nif* genes in plant?, in *Basic Life Sciences,* Vol. 9, Hollaender, A., Burris, R. H., Day, P. R., Hardy, R. W. F., Helinski, D. R., Lamborg, M. R., Owens, L., and Valentine, R. C., Eds., Plenum Press, New York, 1977, 159.
36. **Kameneva, S. V.,** Potential uses of plasmids for facilitating of nitrogen fixation, *Nauchn. Dokl. Vyssh. Shk. Biol. Nauki,* 7, 5, 1980 (in Russian).
37. **Shaw, C. H.,** Genetic engineering of crop plants: a strategy for the future, and the present, *Chem. Ind.,* 23, 809, 1984.
38. **Carlson, P. S. and Chaleff, R. S.,** Forced association between higher plant and bacterial cells *in vitro, Nature (London),* 252, 393, 1974.
39. **Burgoon, A. C. and Bottino, P. J.,** Uptake of the nitrogen fixing blue-green algae *Gloeocapsa* into protoplasts of tobacco and maize, *J. Hered.,* 67, 223, 1976.
40. **Vasil, I. K., Vasil, V., and Hubbell, D. H.,** Engineered plant cell or fungal association with bacteria that fix nitrogen, in *Basic Life Sciences,* Vol. 9, Hollaender, A., Burris, R. H., Day, P. R., Hardy, R. W. F., Helinski, D. R., Lamborg, M. R., Owens, L., and Valentine, R. C., Eds., Plenum Press, New York, 1977, 197.
41. **Stewart, W. D. P., Rowell, P., and Rai, A. N.,** Cyanobacteria-eukaryotic plant symbioses, *Ann. Microbiol. (Inst. Pasteur),* 134B, 205, 1983.
42. **Burgoon, A. C. and Bottino, P. J.,** Uptake of the nitrogen fixing blue-green alga *Gloecapsa* by plant protoplasts, in *Basic Life Sciences,* Vol. 9, Hollaender, A., Burris, R. H., Day, P. R., Hardy, R. W. F., Helinski, D. R., Lamborg, M. R., Owens, L., and Valentine, R. C., Eds., Plenum Press, New York, 1977, 213.
43. **Burris, R. H.,** Overview of nitrogen fixation, in *Basic Life Sciences,* Vol. 9, Hollaender, A., Burris, R. H., Day, P. R., Hardy, R. W. F., Helinski, D. R., Lamborg, M. R., Owens, L., and Valentine, R. C., Eds., Plenum Press, New York, 1977, 9.
44. **Fogg, G. E., Stewart, W. D. P., Fay, P., and Walsby, A. E.,** *The Blue-Green Algae,* Academic Press, London, 1973.
45. **Wolk, C. P.,** Physiology and cytological chemistry of blue-green algae, *Bacteriol. Rev.,* 1973, 37, 1973.
46. **Shah, A. K. and Vaidya, B. S.,** Detection of vitamin B_{12} and pantothenic acid in cell exudates of blue-green algae, *Biol. Plant.,* 19, 426, 1977.
47. **Gibson, C. E. and Smith, R. V.,** Freshwater plankton, in *Botanical Monographs,* Vol. 19, Carr, N. G. and Whitton, B. A., Eds., Blackwell Scientific, Oxford, 1982, 464.
48. **Pearl, H. W.,** Interactions with bacteria, in *Botanical Monographs,* Vol. 19, Carr, H. G. and Whitton, B. A., Eds., Blackwell Scientific, Oxford, 1982, 441.
49. **Suleimanova, Sh. S. and Mineyeva, L. A.,** Effects of high light intensity upon growth and pigment content of cyanobacteria under conditions of photoauto- and photoheterotrophic cultivation, *Vestn. Mosk. Univ. Ser. Biol.,* 1, 42, 1981 (in Russian).
50. **Street, H. E.,** Cell (suspension) cultures-techniques, in *Botanical Monographs,* Vol. 11, 2nd ed., Street, H. E., Ed., Blackwell Scientific, Oxford, 1977, 61.
51. **Holsten, R. D. and Hardy, R. W.,** N_2-fixing plant-bacterial symbiosis in tissue culture, in *Methods Enzymol.,* Vol. 24, 1972, 497.
52. **Stanier, R. Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G.,** Purification and properties of unicellular blue-green algae (order *Chroococcales), Bacteriol. Rev.,* 35, 171, 1971.
53. **Anderson, O. R.,** The radiolarion symbiosis, in *Algal Symbiosis,* Goff, L. J., Ed., Cambridge University Press, Cambridge, 1983, 69.
54. **Evans, P. K. and Cocking, E. C.,** Isolated plant protoplasts, in *Botanical Monographs,* Vol. 11, 2nd ed., Street, H. E., Ed., Blackwell Scientific, Oxford, 1977, 103.
55. **Davey, M. R. and Cocking, E. C.,** Uptake of bacteria by isolated higher plant protoplasts, *Nature (London),* 239, 455, 1972.
56. **Davey, M. R. and Power, J. B.,** Polyethylene glycol-induced uptake of microorganisms into higher plant protoplasts: an ultrastructural study, *Plant Sci. Lett.,* 5, 269, 1975.

57. **Hughes, B. G., White, F. G., Vernon, L. P., and Smith, M. A.,** Uptake of cyanelles and blue-green algae into barley and tobacco protoplasts, *Plant Physiol.,* Suppl. 61, 54, 1978.
58. **Meeks, J. C., Malmberg, R. L., and Wolk, C. P.,** Uptake of auxotrophic cells of a heterocyst-forming cyanobacterium by tobacco protoplasts, and the fate of their associations, *Planta,* 139, 55, 1978.
59. **Bradley, P. M.,** Micromanipulation of cyanelles and a cyanobacterium into higher plant cells, *Physiol. Plant.,* 46, 293, 1979.
60. **Bradley, P. M.,** Micromanipulation and polyethylene glycol induced uptake of cyanelles into plant cells, in *Endocytobiology,* Vol. 1, Schwemmler, W. and Schenk, H. E. A., Eds., Walter de Gruyter, Berlin, 1980, 515.
61. **Bradley, P. M.,** *In vitro* models for plant development, *Dissertation Abstracts International,* Vol. 44, Order No. DA 8407038, Ann Arbor, Mich., 1984.
62. **Bradley, P. M. and Leith, A.,** Uptake of cyanobacteria contained in oil drops by plant protoplasts, *Naturwissenschaften,* 66, 111, 1979.
63. **Fowke, L. C., Greeshoff, P. M., and Marchant, H. J.,** Transfer of organelles of the alga *Chlamydomonas reinhardii* into carrot cells by protoplast fusion, *Planta,* 144, 341, 1979.
64. **Fowke, L. C., Marchant, H. J., and Gresshoff, P. M.,** Fusion of protoplasts from carrot cell cultures and the green alga *Stigeoclonium, Can. J. Bot.,* 59, 1021, 1981.
65. **Agafodorova, M. N., Gorelova, O. A., Baulina, O. I., Semionova, L. R., Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.,** The study of the initial stages of the cyanobacteria cells' and spheroplasts' penetration into the isolated protoplasts of tobacco, induced by polyethylene glycol, *Izv. Akad. Nauk SSSR Ser. Biol.,* 4, 510, 1982 (in Russian).
66. **Wolk, C. P. and Shaffer, P. W.,** Heterotrophic micro- and macrocultures of a nitrogen-fixing cyanobacterium, *Arch. Microbiol.,* 110, 145, 1976.
67. **Gusev, M. V., Mineyeva, L. A., and Semenova, L. R.,** The effect of lysozyme, EDTA, magnesium and mannitol on spheroplast formation by *Anacystis nidulans, Mikrobiologiya,* 48, 693, 1979 (in Russian).
68. **Semenova, L. R., Mineyeva, L. A. and Gusev, M. V.,** The effect of osmotic stabilizing agents on the formation of spheroplasts in cyanobacteria and their photosynthetic activity, *Mikrobiologiya,* 51, 259, 1982 (in Russian).
69. **Kao, K. N. and Michayluk, M. R.,** A method for high-frequency intergeneric fusion of plant protoplasts, *Planta,* 115, 355, 1974.
70. **Trench, R. K., Pool, R. R., Logan, J. M., and Engeland, A.,** Aspects of the relation between *Cyanophora paradoxa* (Korschikoff) and its endosymbiotic cyanelles *Cyanocyta Korsschikoffiana* (Hall a. Claus). I. Growth, ultrastructure, photosynthesis and the obligate nature of the association, *Proc. R. Soc. London,* 202, 423, 1978.
71. **Bonnett, H. and Eriksson, T.,** Transfer of algal chloroplasts into protoplasts of higher plants, *Planta,* 120, 71, 1974.
72. **Nagata, T.,** A novel cell-fusion method of protoplasts by polyvinyl alcohol, *Naturwissenschaften,* 65, 263, 1978.
73. **Hasezawa, S., Matsui, C., Nagata, T., and Syono, K.,** Cytological study of the introduction of *Agrobacterium tumefaciens* spheroplasts into *Vinca rosea* protoplasts, *Can. J. Bot.,* 61, 1052, 1983.
74. **Matsui, C., Hasezawa, S., Tanaka, M., and Syono, K.,** Introduction of *Escherichia coli* cells and spheroplasts into *Vinca* protoplasts, *Plant Cell Rep.,* 2, 30, 1983.
75. **Keller, W. A. and Melchers, G.,** The effect of high pH and calcium on tobacco leaf protoplast fusion, *Z. Naturforsch.,* 28, 737, 1973.
76. **Agafodorova, M. N., Baulina, O. I., Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.,** Use of calcium ions at high pH for production of the association of cyanobacteria and isolated tobacco protoplasts, *Dokl. Akad. Nauk SSSR,* 265, 758, 1982 (in Russian).
77. **Baulina, O. I., Agafodorova, M. N., Korzhenevskaya, T. G., Gusev, M. V., and Butenko, R. G.,** An artificial association of the cyanobacterium and tobacco callus tissue, *Mikrobiologiya,* 53, 997, 1984 (in Russian).
78. **Berg, H., Jacob, H. E., and Mühlig, P.,** Zell Verschmelzung durch hoch Spannung, *Urania,* 60, 68, 1984.
79. **Zimmerman, U. and Scheurich, P.,** High frequency fusion of plant protoplasts by electric fields, *Planta,* 151, 26, 1981.
80. **Veliky, I. and LaRue, T. A.,** Changes in soybean root culture induced by *Rhizobium japonicum, Naturwissenschaften,* 54, 96, 1967.
81. **Holsten, R. D., Burns, R. C., Hardy, R. W., and Hebert, R. R.,** Establishment of symbiosis between *Rhizobium* and plant cells *in vitro, Nature (London),* 232, 173, 1971.
82. **Rao, V. R., Sopory, S., and Rao, N. S. S.,** Establishment of symbiosis *in vitro* between *Rhizobium* and pea (*Pisum sativum*) root callus, *Curr. Sci.,* 43, 503, 1974.
83. **Khailova, G. F., Mamedov, K. Yu., Shilnikova, V. K., and Ilyasova, V. B.,** Symbiotic association of alfalfa root calli with *Rhizobium meliloti, Izv. Timiryazevsk. Skh. Akad.,* 4, 18, 1977 (in Russian).

84. **Child, J. J.,** Nitrogen fixation by a *Rhizobium* sp. in association with non-leguminous plant cell culture, *Nature (London),* 253, 350, 1975.
85. **Scowcroft, W. R. and Gibson, A. H.,** Nitrogen fixation by *Rhizobium* associated with tobacco and cowpea cell cultures, *Nature (London),* 253, 351, 1975.
86. **Yurkova, G. N., Nichik, M. M., Levenko, B. A., and Starchenkov, E. P.,** Fixation of molecular nitrogen by nodule bacteria in association with tobacco and wheat cells, *Dokl. Akad. Nauk SSSR,* 230, 1006, 1976 (in Russian).
87. **Vasil, V., Vasil, I. K., Zuberer, D. A., and Hubbell, D. H.,** The biology of *Azospirillum*-sugarcane association. I. Establishment of the association *in vitro. Z. Pflanzenphysiol.,* 95, 141, 1979.
88. **Vasil, I. K., Vasil, V., White, D. W. R., and Berg, R. H.,** Plant tissue cultures in plant improvement, in *Developments in Plant Biology,* Vol. 5, Sala, F., Ed., Biomedical Press, Amsterdam, 1980, 215.
89. **Berg, R. H., Vasil, V., and Vasil, I. K.,** The biology of *Azospirillum*-sugarcane association. II. Ultrastructure, *Protoplasma,* 101, 143, 1979.
90. **Berg, R. H., Tyler, M. E., Novick, N. J., Vasil, V., and Vasil, I. K.,** Biology of *Azospirillum*-sugarcane association: enhancement of nitrogenase activity, *Appl. Environ. Microbiol.,* 39, 642, 1980.
91. **Child, J. J. and Kurz, W. G. W.,** Inducing effect of plant cells on nitrogenase activity by *Spirillum* and *Rhizobium in vitro, Can. J. Microbiol.,* 24, 143, 1978.
92. **Kurz, W. G. W. and Child, J. J.,** Inducing effect of plant cells on nitrogenase activity by *Spirillum* and *Rhizobium in vitro,* in Abstr. 12th Int. Congr. Microbiol., Munich, 1978, E72.
93. **Bradley, P. M.,** Co-culture of carrot cells and a green alga on medium deficient in nitrogen, *Z. Pflanzenphysiol.,* 100, 65, 1980.
94. **Bradley, P. M.,** Induced *in vitro* symbiosis using algae and callus of *Daucus carota* on medium deficient in nitrogen, in *Endocytobiology,* Vol. 2, Schwemmler, W. and Schenk, H. E. A., Eds., Walter de Gruyter, Berlin, 1983, G13.
95. **Wolters, B. and Eilert, U.,** Acridonepoxidgehalte in Kalluskulturen vol *Ruta graveolens* und ihre Steigerund durch Mischkultur mit Pilzen, *Z. Naturforsch.,* 37c, 575, 1982.
96. **Hardy, R. W. F., Holsten, R. D., Jackson, E. K., and Burns, R. C.,** The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation, *Plant Physiol.,* 43, 1185, 1968.
97. **Reporter, M.,** Synergetic cultures of *Glycine max* root cells and *Rhizobia* separated by membrane filters, *Plant Physiol.,* 57, 651, 1976.
98. **Lustig, B., Plischke, W., and Hess, D.,** Induction of nitrogenase activity in associations between *Portulaca grandiflora* and *Rhizobium*: a simple transfilter apparatus for studying nitrogen fixation in associations between non-leguminous plant cell cultures and rhizobia on solid media, *Z. Pflanzenphysiol.,* 98, 277, 1980.
99. **Hess, D. and Lustig, B.,** Transfilter associations of nonleguminous plants with rhizobia: channeling of N_2 fixed by rhizobia into the nitrogen metabolism of *Portulaca grandiflora* callus tissues, *Experientia,* 37, 475, 1981.
100. **Rao, V. R. and Rao, N. S. S.,** Studies on the interaction of legumine root callus with *Rhizobium, Z. Pflanzenphysiol.,* 80, 14, 1976.
101. **Gupta, R. P., Kalra, M. S., and Bajaj, Y. P. S.,** Nitrogen fixation in cell cultures of some legumes and nonlegumes, *Indian J. Exp. Biol.,* 22, 560, 1984.
102. **Ozawa, T. and Yamaguchi, M.,** Increase in cellulase activity in cultured soybean cells caused by *Rhizobium japonicum, Plant Cell Physiol.,* 21, 331, 1980.
103. **Khailova, G. F.,** A study of the mechanisms of establishment of an efficient symbiosis on the systems: alfalfa tissue culture — mutant rhizobia strains, in *Abstr. 4th All-Union Conf. Plant Cell Culture and Biotechnology,* Štiinca, Kishinev, 1983, 180 (in Russian).
104. **Child, J. J. and LaRue, T. A.,** A simple technique for the establishment of nitrogenase in soybean callus culture, *Plant Physiol.,* 53, 88, 1974.
105. **Schetter, C. and Hess, D.,** Nitrogenase activity in *in vitro* associations between callus tissues of non-leguminous horticultural plants and *Rhizobium, Plant Sci. Lett.,* 9, 1, 1977.
106. **Reporter, M.,** Synergetic cultures of *Glycine max* root cells and rhizobia separated by membrane filters, *Plant Physiol.,* 57, 651, 1976.
107. **Patriquin, D. G., Döbereiner, J., and Jain, D. K.,** Sites and processes of association between diazotrophs and grasses, *Can. J. Microbiol.,* 29, 800, 1983.
108. **Pence, V. C., Novic, N. J., Ozias-Akins, P., and Vasil, I. K.,** Induction of nitrogenase activity in *Azospirillum brasilense* by conditioned medium from suspension cultures of *Pennisetum americanum* (pearl millet) and *Panicum maximum* (Guinea grass), *Z. Pflanzenphysiol.,* 106, 139, 1982.
109. **Goff, L. J., Ed.,** *Algal Symbiosis,* Cambridge University Press, Cambridge, 1983.
110. **Rokem, J. S., Schwarzberg, J., and Goldberg, I.,** Autoclaved fungal mycelia increase diosgenin production in cell suspension cultures of *Dioscorea deltoidea, Plant Cell Rep.,* 3, 159, 1984.
111. **Butenko, R. G., Gusev, M. V., Korzhenevskaya, T. G., and Yagodina, I. B.,** Cultivation of the tobacco cell and cyanobacterium association, in *Abstr. 3rd All-Union Conf. Plant Cell Culture,* Butenko, R. G., Ed., Academia Nauk Armenian SSR, Abovyan, 1979, 40 (in Russian).

112. **Gusev, M. V., Butenko, R. G., Korzhenevskaya, T. G., and Yagodina, I. B.,** Associative culture of tobacco (*Nicotiana tabacum*) and cyanobacterium (*Anacystis nidulans*) cells, *Fiziol. Rast.*, 29, 550, 1982 (in Russian).
113. **Gusev, M. V. and Korzhenevskaya, T. G.,** Cyanobacteria in associations with higher plant cells, in *Abstr. 4th Int. Symp. Photosynthetic Prokaryotes,* Bombannes, France, 1982, E 1.
114. **Yagodina, I. B.,** The dynamics of growth processes in associative culture of tobacco cells and cyanobacterium, in *Abstr. 4th All-Union Conf. Plant Cell Culture and Biotechnology,* Butenko, R. G., Ed., Štiinca, Kishinev, 1983, 182 (in Russian).
115. **Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.,** Introduction of photosynthesizing and nitrogen-fixing symbionts into plant cell cultures and regenerants, in *Proc. Int. Symp. Plant Tissue and Cell Culture; Application to Crop Improvement,* Novak, F. J., Havel, L., and Doležel, J., Eds., Czechoslovak Academy of Sciences, Prague, 1984, 427.
116. **Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.,** Potential uses of associative cell and tissue cultures in biotechnology, *Nauchn. Dokl. Vyssh. Shk. Biol. Nauki,* 12, 46, 1985 (in Russian).
117. **Gusev, M. V., Butenko, R. G., Korzhenevskaya, T. G., Lobacova, E. S., and Baulina, O. I.,** Intercellular symbiosis of suspension ginseng culture cells and cyanobacteria, *Eur. J. Cell Biol.*, 22, 503, 1980.
118. **Butenko, R. G., Gusev, M. V., Korzhenevskaya, T. G., and Lobacova, E. S.,** Growth of the associative culture of ginseng (*Panax ginseng*) cells and cyanobacterium (*Chlorogloea fritschii*) in the light and darkness, *Fiziol. Rast.*, 29, 995, 1982 (in Russian).
119. **Baulina, O. I. and Lobacova, E. S.,** An electron-microscopic study of ginseng cells and cyanobacteria in mixed culture, in *Abstr. 4th All-Union Conf. Plant Cell Culture and Biotechnology,* Štiinca, Kishinev, 1983, 182 (in Russian).
120. **Lobacova, E. S. and Sizov, A. D.,** The regulatory action of oscillatory changes in pH and pO_2 on the functioning of the association of ginseng and cyanobacterial cells, in *Abstr. 4th All-Union Conf. Plant Cell Culture and Biotechnology,* Štiinca, Kishinev, 1983, 181 (in Russian).
121. **Korzhenevskaya, T. G., Lobacova, E. S., Ilyash, L. V., Butenko, R. G., and Gusev, M. V.,** Photosynthetic products of the cyanobacterium *Chlorogloea fritschii* are used by ginseng cells, *Dokl. Akad. Nauk SSSR,* 276, 765, 1984 (in Russian).
122. **Lobacova, E. S., Korzhenevskaya, T. G., Gusev, M. V., and Butenko, R. G.,** Production of biologically active substances by ginseng cells in the association with cyanobacterium *Chlorogloea fritschii, Dokl. Akad. Nauk SSSR,* 276, 1017, 1984 (in Russian).
123. **Korzhenevskaya, T. G., Gorelova, O. A., Gusev, M. V., and Butenko, R. G.,** Cocultivation of higher plant cells and nitrogen-fixing cyanobacteria, *Fiziol. Rast.*, 32, 88, 1985 (in Russian).
124. **Korzhenevskaya, T. G. and Gusev, M. V.,** Artificial syncyanoses, in *Abstr. 5th Int. Symp. Photosynthetic Prokaryotes,* Grindelwald, Switzerland, September 22—28, 1985, 146.
125. **Baulina, O. I. and Lobacova, E. A.,** Morphology and ultrastructure of ginseng-cyanobacterium association in suspension cultures, in *Plant Cell Culture and Biotechnology,* Butenko, R. G., Ed., Nauka, Moscow, 1986, 252 (in Russian).
126. **Korzhenevskaya, T. G., Lobacova, E. S., Sizov, A. D., Butenko, R. G., and Gusev, M. V.,** Changes in physicochemical medium factors in ginseng and cyanobacterium cell association, *Izv. Akad. Nauk SSSR Ser. Biol.*, 1, 5, 1986 (in Russian).
127. **Lobacova, E. S. and Baulina, O. I.,** Ultrastructure of the suspension ginseng cell culture assessed for subsequent formation of the association with cyanobacteria, in *Plant Cell Culture and Biotechnology,* Butenko, R. G., Ed., Nauka, Moscow, 1986, 246 (in Russian).
128. **Lobacova, E. S. and Korzhenevskaya, T. G.,** Effect of the medium composition on the growth of a ginseng cell association with the cyanobacterium *Chlorogloeopsis fritschii, Vestn. Mosk. Univ. Ser. Biol.*, 1, 31, 1986 (in Russian).
129. **Gusev, M. V., Korzhenevskaya, T. G., and Butenko, R. G.,** Experimentally produced associations of higher plant cultivated cells, tissues and regenerants with cyanobacteria, in *Abstr. 16th Meet. Fed. European Biochemical Societies,* FEBS, Moscow, 1984, 237 (in Russian).
130. **Korzhenevskaya, T. G., Skripnikov, A. Yu., Butenko, R. G., and Gusev, M. V.,** Artificial associations of alfalfa cells and plants with a nitrogen-fixing cyanobacterium, *Dokl. Akad. Nauk SSSR,* 284, 765, 1985 (in Russian).
131. **Skripnikov, A. Yu. and Korzhenevskaya, T. G.,** The stimulatory effect of plant cells on cyanobacterial nitrogenase activity *in vitro,* in *Plant Cell Culture and Biotechnology,* Butenko, R. G., Ed., Nauka, Moscow, 1986, 225 (in Russian).
132. **Gorelova, O. A., Řeřabek, J., Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.,** The growth and biosynthetic activity of *Solanum laciniatum* cells in mixed cultures with nitrogen-fixing cyanobacteria, *Dokl. Akad. Nauk SSSR,* 279, 253, 1984 (in Russian).
133. **Gorelova, O. A., Řeřabek, J., Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.,** Influence of the cyanobacterium *Chlorogloeopsis fritschii* on growth and biosynthetic activity of *Solanum laciniatum* cells in mixed culture, *Fiziol. Rast.*, 32, 1158, 1985 (in Russian).

134. **Gusev, M. V. and Korzhenevskaya, T. G.**, Introduction of cyanobacteria into cell cultures and regenerates of higher plants, in *Abstr. 5th Int. Symp. Photosynthetic Prokaryotes*, Grindelwald, Switzerland, September 22—28, 1985, 145.

135. **Gorelova, O. A.**, Biosynthesis and accumulation of steroid compounds by *Solanum laciniatum* cells under conditions of mixed culturing with cyanobacteria, in *Plant Cell Culture and Biotechnology*, Butenko, R. G., Ed., Nauka, Moscow, 1986, 261 (in Russian).

136. **Korzhenevskaya, T. G., Pyvovarova, L. V., and Baulina, O. I.**, Associated with cyanobacteria growth of tobacco cultivated tissues and plants, in *Abstr. 4th All-Union Conf. Plant Cell Culture and Biotechnology*, Štiinca, Kishinev, 1983, 179 (in Russian).

137. **Korzhenevskaya, T. G., Pyvovarova, L. V., Baulina, O. I., Butenko, R. G., and Gusev, M. V.**, Associations of tobacco regenerates with nitrogen-fixing cyanobacteria, *Dokl. Akad. Nauk SSSR*, 274, 1016, 1984 (in Russian).

138. **Pyvovarova, L. V., Korzhenevskaya, T. G., Baulina, O. I., Butenko, R. G., and Gusev, M. V.**, Use of different methods for the introduction of a nitrogen-fixing cyanobacterium into tobacco plants, *Izv. Akad. Nauk SSSR Ser. Biol.*, 5, 645, 1985 (in Russian).

139. **Gusev, M. V., Korzhenevskaya, T. G., Pyvovarova, L. V., Baulina, O. I., and Butenko, R. G.**, Introduction of a nitrogen-fixing cyanobacterium into tobacco shoot regenerates, *Planta*, 167, 1, 1986.

140. **Pyvovarova, L. V., Korzhenevskaya, T. G., Butenko, R. G., and Gusev, M. V.**, Localization of cyanobacteria, growing in association with callus culture and with regenerated plants of tobacco, *Fiziol. Rast.*, 33, 74, 1986 (in Russian).

141. **Trukhanov, V. A., Checheneva, T. N., Shulzhenko, T. P., Ruban, T. A., and Kordyum, V. A.**, Production of associative system: blue-green algae-tobacco tissue culture, in *Abstr. 4th All-Union Conf. Plant Cell Culture and Biotechnology*, Štiinca, Kishinev, 1983, 183 (in Russian).

142. **Kordyum, V. A., Trukhanov, V. A., Checheneva, T. N., Ruban, T. A., and Shulzhenko, T. P.**, Artificial nitrogen-fixing system "tobacco tissue culture — blue-green algae", in *Abstr. Int. Symp. Plant Tissue and Cell Culture; Application to Crop Improvement*, Olomouz, Czechoslovakia, 1984, 90.

143. **Gusev, M. V. and Korzhenevskaya, T. G.**, Growth of callus cultures and plants inoculated with nitrogen-fixing cyanobacterium *Anabaena variabilis, Abstr. 14th Int. Congr. Microbiology*, Manchester, England, September 7th to 13th, 1986, 233.

144. **Gusev, M. V., Butenko, R. G., Agafodorova, M. N., Korzhenevskaya, T. G., and Baulina, O. I.**, Morphogenesis in mixed callus culture of tobacco and cyanobacterium, *Dokl. Akad. Nauk SSSR*, 272, 1007, 1982 (in Russian).

145. **Pyvovarova, L. V. and Gusev, M. V.**, Introduction of a nitrogen-fixing cyanobacterium into tobacco plant, in *Plant Cell Culture and Biotechnology*, Butenko, R. G., Ed., Nauka, Moscow, 1986, 242 (in Russian).

146. **Lipsky, A. Kh., Nosov, A. M., Paukov, V. N., and Karanova, S. L.**, Growth and metabolism of the *Dioscorea deltoidea* cell culture on submerged cultivation, in *Plant Cell Culture, Advances in Science and Technology in the USSR*, Butenko, R. G., Ed., Mir Publishers, Moscow, 1985, 76.

147. **Shamina, Z. B., Frolova, L. V., Paukov, V. N., and Sovalova, L. A.**, The study of the poppy cell culture, in *Abstr. 3rd All-Union Conf. Plant Cell Culture*, Butenko, R. G., Ed., Akademia Nauk Armenian SSR, Abovyan, 1979, 72 (in Russian).

148. **Řeřabek, J. and Gorelova, O. A.**, The dependence of the growth and steroid accumulation on the type and amount of nitrogen compounds in the suspension culture of *Solanum laciniatum*, in *Plant Cell Culture and Biotechnology*, Butenko, R. G., Ed., Nauka, Moscow, 1986, 70 (in Russian).

149. **Padhy, S. N. and Pattnaik, H.**, Effect of IAA on growth and nitrogen fixation of *Westiellopsis prolifica* Janet., *Z. Pflanzenphysiol.*, 75, 460, 1975.

150. **von Wettstein, D. and Eriksson, T.**, The genetics of chloroplasts, in *Genetics Today*, Vol. 3, Geerts, S. J. and Abeelen, J. H. F. V., Eds., Pergamon Press, Oxford, 1965, 591.

151. **Murashige, T. and Skoog, F.**, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plant.*, 15, 473, 1962.

152. **Ahmadjian, V. and Jacobs, J. B.**, Algal-fungal relationships in lichens: recognition, synthesis and development, in *Algal Symbiosis*, Goff, L. J., Ed., Cambridge University Press, Cambridge, 1983, 147.

153. **Rodgers, G. A.**, The effect of some external factors on nitrogenase activity in free-living and endophytic *Nostoc* of the liver wort *Blasia pusilla, Physiol. Plant.*, 44, 407, 1978.

154. **Silvester, W. B. and McNamara, P. J.**, The infection process and ultrastructure of the *Gunnera-Nostoc* symbiosis, *New Phytol.*, 77, 135, 1976.

155. **Grilli-Caiola, M.**, On the phycobionts of the cycad coralloid roots, *New Phytol.*, 85, 537, 1980.

156. **Obukowicz, M., Schaller, M., and Kennedy, G. S.**, Ultrastructure and phenolic histochemistry of the *Cycas revoluta-Anabaena* symbiosis, *New Phytol.*, 87, 751, 1981.

157. **Flower, M. V.**, Substrate utilization by plant-cell cultures, *J. Chem. Technol. Biotechnol.*, 32, 338, 1982.

158. **Korzhenevskaya, T. G. and Gusev, M. V.**, Metabolism and destruction of the blue-green alga *Anabaena variabilis* in the dark, *Mikrobiologiya*, 45, 795, 1976 (in Russian).

159. **Pilet, P. E. and Bernasconi, P.,** *Rubus hispidus* callus cultured *in vitro* and endogenous lysozyme: growth processes, *Plant Sci. Lett.,* 35, 147, 1984.
160. **Jhong, J. J., Staba, E. J., and Kim, J. Y.,** American and Korean ginseng tissue cultures: growth, chemical analysis and plantlet production, *In Vitro,* 9, 253, 1974.
161. **Butenko, R. G., Khretonova, T. I., Slepian, L. I., Mikhailova, N. V., and Vysotzkaya, R. I.,** Phytochemical analysis of the strain of tissue culture of *Panax ginseng* C. A. Mey. and standardization of its preparation, *Rastit. Resur.,* 15, 356, 1979 (in Russian).
162. **Tso, W. W. and Fung, W. P.,** Stimulation of bacterial oxygen consumption by ginseng root extract, *Microbios Lett.,* 13, 7, 1980.
163. **Allen, M. B. and Arnon, D. S.,** Studies on nitrogen-fixing blue-green algae. II. The sodium requirement of *Anabaena cylindrica, Physiol. Plant.,* 8, 653, 1955.
164. **Gamborg, O. L., Miller, R. A., and Ojima, K.,** Nutrient requirements of suspension cultures of soybean root cells, *Exp. Cell Res.,* 50, 151, 1968.
165. **Natanielsz, C. P. and Staff, I. A.,** On the occurrence of intracellular blue-green algae in cortical cells of the apogeotropic roots of *Macrozamia communis* L. Johnson, *Ann. Bot. (London),* 39, 363, 1975.
166. **Peters, G. A.,** The *Azolla-Anabaena azollae* symbiosis, in *Basic Life Sciences,* Vol. 9, Hollaender, A., Burris, R. H., Day, P. R., Hardy, R. W. F., Helinski, D. R., Lamborg, M. R., Owens, L., and Valentine, R. C., Eds., Plenum Press, New York, 1977, 231.
167. **Fay, P. and Kulassoriya, S. A.,** Tetrazolium reduction and nitrogenase activity in heterocystous blue-green algae, *Arch. Mikrobiol.,* 87, 341, 1972.
168. **Peters, G. A. and Mayne, B. C.,** The *Azolla-Anabaena azollae* relationship. II. Localization of nitrogenase activity as assayed by acetylene reduction, *Plant Physiol.,* 53, 820, 1974.
169. **Bhaskaran, J. and Venkataraman, G. J.,** Occurrence of a blue-green alga in the nodules of *Trifolium alexandrinum, Nature (London),* 181, 277, 1958.
170. **Pyvovarova, L. V., Korzhenevskaya, T. G., Ilyash, L. V., Butenko, R. G., and Gusev, M. V.,** Uptake of tobacco metabolites by cyanobacteria in artificial association, *Dokl. Akad. Nauk SSSR,* 297, 766, 1987.
171. **Butenko, R. G. and Volodarsky, A. D.,** Specificity of antigens in the cycle of cellular transformations in a tobacco tissue culture, *Fiziol. Rast.,* 14, 956, 1967 (in Russian).
172. **Butenko, R. G., Volodarsky, A. D., and Moiseeva, N. A.,** Differentiation of plant cells and its protein expression, *Eur. J. Cell Biol.,* 22, 455, 1980.
173. **Moiseeva, N. A., Volodarsky, A. D., and Butenko, R. G.,** Detection of antigen markers in tobacco stem meristem, *Fiziol. Rast.,* 26, 479, 1979 (in Russian).
174. **Gates, J. E., Fisher, R. W., Goggin, T. W., and Azrolan, N. A.,** Antigenic differences between *Anabaena azollae* fresh from the *Azolla* fern leaf cavity and free-living cyanobacteria, *Arch. Microbiol.,* 128, 126, 1980.

Chapter 9

GENERAL METHODS

Amar N. Rai

Presented in this chapter are some general procedures for maintaining cyanobacterial symbioses, separation of the symbionts, and their cultivation.

LICHENS

Lichens are very sensitive to pollution and environmental changes. For experimental purposes, samples are generally collected from the field and maintained in a growth chamber under desirable experimental conditions.

Whole lichen thalli or discs are used for studying the association. For studying the cyanobiont, thalli can be treated with digitonin (0.01%, w/v) which disrupts fungal membranes but does not affect the cyanobiont cells. It is not possible to isolate large quantities of clean cyanobiont cells from lichen thalli for direct experimentation.

To study the cyanobiont in culture, cyanobacteria are isolated from surface-sterilized lichen thalli by crushing a piece of the lichen thallus and spreading it on nutrient agar plates. Cyanobacterial colonies are picked up from such plates and axenic cultures of the cyanobacterium raised using standard microbiological techniques. Following is the detail of isolating and culturing the symbiotic cyanobacterium (*Nostoc*) from *Peltigera aphthosa.*[1]

Lichen thalli collected from the field are brought to the lab, washed with sterilized distilled water, and surface sterilized by dipping in 3% sodium hypochlorite solution for 30 s. The cephalodia are immediately excised and washed in sterilized distilled water. These cephalodia are then squashed, using sterilized needles, on a microscope slide (sterilized) and plated on BG-11_0 agar plates.[2] The plates are then incubated at 22°C and under 1000 lx continuous light. When colonies develop on the plate, a single cyanobacterial colony is picked up and spread on another plate. The isolated cyanobacterial filaments are then freed from bacteria using a modification of the procedure described by Bunt.[3] A 3-cm layer of BG-11_0 agar (BG-11 medium without nitrate) is poured into 15 × 1.5-cm test tube and sterilized by autoclaving. A small piece of agar containing the cyanobacterial colony is then cut out aseptically from the petri dish and placed on top of the BG-11_0 agar in the test tube. Another layer (2 cm deep) of sterilized BG-11_0 agar containing 10 μg/ml polymixin-B-sulfate and 100 μg/ml cycloheximide is poured on top of the inoculum and allowed to set. Finally, a 2-cm-deep layer of BG-11_0 liquid medium is poured on top of this solid layer and the tube plugged with cotton wool. These tubes are incubated in the dark for 24 h and then transferred to light (3000 lx) at 22°C. Initial dark incubation stimulates production of harmogonia which migrate through the antibiotic containing agar and reach the top liquid layer during light incubation. Most of the bacteria remain in the sheath, while some bacterial or eukaryotic contaminants which remain attached to the harmogonia are killed by polymixin-B-sulfate and cycloheximide, respectively. This procedure is repeated until axenic cultures result.

Stock cultures of the cyanobacterium are obtained by removing aliquots of liquid BG-11o (0.1 ml) from the tubes with a sterile syringe and plating them on sterile BG-11o agar in petri dishes which are incubated at 22°C and 3000-lx light intensity. These cultures are checked regularly for contaminants by microscopic observation and by plating a small piece of material on nutrient agar plates and used for further study. The stocks are maintained on agar slants in screw-capped tubes. Large volume liquid cultures can be raised by inoculating liquid BG-11o medium with an inoculum from the stock.

TABLE 1
Composition of Proskauer Solution

Compounds	Concentration
$MgSO_4 \cdot 7H_2O$	100 mg/l
$CaCl_2$	100 mg/l
KH_2PO_4	100 mg/l
$FeSO_4$	50 mg/l
KNO_3	250 mg/l[a]
$LiCl_2$	28 μg/l
$CuSO_4$	56 μg/l
$ZnSO_4$	56 μg/l
H_3BO_3	611 μg/l
Al_2SO_4	56 μg/l
$SnCl_2 \cdot 2H_2O$	28 μg/l
KBr	28 μg/l
$MnCl_2 \cdot 4H_2O$	389 μg/l
$NiSO_4 \cdot 6H_2O$	56 μg/l
$Co(SO_4)_2 \cdot 6H_2O$	56 μg/l
TiO_2	56 μg/l
KI	28 μg/l

[a] Used only for the *Nostoc*-free bryophyte symbiont.

LIVERWORTS AND HORNWORTS

Among bryophyte-cyanobacterial symbioses, most of the physiological and biochemical work has been done on *Anthoceros* and *Blasia*. To maintain the thalli in the laboratory, field-collected samples are washed with sterilized water and then placed on peralite, moistened with distilled water, in shallow plastic trays with perspex lids (inside light intensity 200 lx) at 20°C. The thalli are kept moist with regular sprays of nitrogen-free Proskaur solution (Table 1) containing trace elements.[4]

Axenic thalli can be obtained as needed by using the method of Enderlin and Meeks.[5] They have obtained and cultured *Anthoceros* gametophyte by germinating surface-sterilized spores. Mature sporophytes are excised, treated with 0.5% sodium hypochlorite and 0.1% detergent for 0.5 to 2 min, and then washed with sterilized distilled water. The sporophyte is split lengthwise and spores are spread over the basal agar medium consisting 0.2 g/l ammonium nitrate; 0.1 g/l each of calcium chloride, magnesium sulfate, and potassium dihydrogen phosphate; 1 ml/l micronutrient solution;[6] and 1.2% agar (pH adjusted to 6.4 before autoclaving). The plates are incubated at 20°C under a 14/10-h light-dark cycle. As germlings appear, they are transferred to liquid basal medium, plus 1% glucose, buffered with 5 m*M* MES (2-*N*-morpholino ethanesulfonic acid) — NaOH buffer pH 6.4. To obtain axenic *Anthoceros-Nostoc* association, axenic *Anthoceros* thalli (obtained as described above) are reconstituted with axenic *Nostoc* isolate from the association (see later). Reconstitution can be done in liquid cultures as described by Enderlin and Meeks.[5] A population of *Nostoc* cells (up to 9×10^8 cells) and *Anthoceros* gametophyte tissue (multiple pieces; 0.5 to 3 g fresh weight) are incubated together in 50 ml of *Anthoceros* medium lacking combined N. After 2 to 3 weeks of incubation under conditions described for growing *Anthoceros*, the gametophyte tissue is rinsed with sterile medium and transferred to fresh nitrogen-free medium. Appearance of cyanobacterial colonies within the *Anthoceros* tissue indicates the establishment of association. Surface growth of *Nostoc* on gametophyte can be removed by a 2-d incubation in the liquid basal medium supplemented with 250 U/ml of penicillin G, followed by transfer to fresh medium lacking the antibiotic. Reconstitution experiments can also be done on solid media.[4] The method described above is highly dependable and has been successfully utilized in my lab.

The cyanobiont from *Blasia* and *Anthoceros* have been successfully isolated and grown in axenic cultures. A method used by Rodgers[4] is described below.

Cyanobacterial colonies excised from thalli are washed in sterilized distilled water and plated on solid ASM (N-free) medium in petri dishes. The dishes are then incubated at 5000 lx and 30°C for 3 weeks. After cyanobacterial colonies appear on the plates, they are picked up and made bacteria-free by using the method described by Bunt[3] for lichen cyanobionts.

For certain experiments, it may be necessary to obtain symbiotic cyanobacteria from thalli for immediate use. Because of the small thalli, and still smaller proportion of the cyanobiont in it, it is difficult to obtain large quantities of cyanobiont cells for extensive experimentation. However, small amounts of cyanobiont cells can be obtained from thalli using the method described by Meeks et al.,[7] which takes approximately 3 to 4 h. The procedure is as follows.

Anthoceros thalli containing *Nostoc* colonies are suspended in 5 m*M* MOPS (3-*N*-morpholino propane sulfonic acid) — NaOH buffer, pH 7.8, containing polyvinyl pyrrolidone-40 (1%), in 1:1 ratio (w/v). The suspension is blended for 20 s at high speed. The resulting slurry is centrifuged twice at 1000 g in the same buffer and incubated for 15 min at 30°C in the presence of 0.05% (w/v) digitonin. Macerase, cellulase, and β-glucuronidase are then added to the final concentration of 1% (w/v) each and incubated for 1 h. The digested preparation thus obtained is centrifuged twice in 5 m*M* MOPS buffer, pH 7.8, with 2 min vigorous mixing between the centrifugations. The resulting pellet is resuspended in MOPS buffer and loaded on a discontinuous gradient composed of 5% Ficoll-400 (w/v) plus 5% neutralized Ludox AM (colloidal silica) (v/v), 7% Ficoll plus 4% Ludox, 9% Ficoll plus 3% Ludox, and 11% Ficoll plus 2% Ludox. This is then centrifuged at 16,000 g for 10 min at 10°C in a horizontal rotor. Most of the cyanobacterial colonies are banded in the 11% Ficoll plus 2% Ludox region of the gradient. This band is removed carefully and the gradient centrifugation repeated twice. Small amounts of the contaminating undigested material can be removed with the aid of a stereomicroscope (see also Chapter 3).

AZOLLA

Azolla can be grown in the laboratory using the medium described in Table 2. Basal medium with nitrate is used for growing cyanobiont-free *Azolla* and basal medium without nitrate for *Azolla-Anabaena* association.

Field samples of *Azolla* are contaminated with bacteria. To grow them in laboratory, field-collected *Azolla* is thoroughly washed with distilled water and then surface sterilized with 2% Chlorox (or 0.1% sodium hypochlorite) and 0.01% Triton X-100 (as wetting agent) for 20 min. Fronds are then washed thoroughly with sterile distilled water and transferred to sterilized *Azolla* growth medium with or without nitrate. After 1 week, healthy fronds are subcultured in fresh medium.

To obtain *Anabaena*-free *Azolla*, various methods have been suggested. These include sealing *Azolla* in glass-stoppered bottles, giving light stress, or subjecting to antibiotic treatment (see also Chapter 4). Of these, the latter is highly satisfactory and is described below.[8]

Surface-sterilized *Azolla* fronds are transferred to antibiotic-containing *Azolla* growth medium (+nitrate) and maintained for 1 week. These are then transferred to fresh medium containing a different antibiotic. This process is continued until four different combinations (A to D; see below) have been employed. The fronds are eventually transferred to antibiotic-free medium. The following three different sets of antibiotic treatments have been successfully used:

TABLE 2
Composition of the *Azolla* Nutrient Medium

Compounds	Concentration: −Nitrate medium[a]	Concentration: +Nitrate medium[b]
KCl	2.00 mM	—
$CaCl_2$	2.00 mM	—
KNO_3	—	2.00 mM
$Ca(NO_3)_2$	—	2.00 mM
KH_2PO_4	0.40 mM	0.40 mM
$MgSO_4$	0.80 mM	0.80 mM
FeEDTA	6.58 mM	6.58 mM
$Co(NO_3)_2$	0.17 μM	0.17 μM
NH_4VO_3	0.10 μM	0.10 μM
Na_2WO_4	0.05 μM	0.05 μM
$TiO\ (Cr_2O_4)_x$	0.05 μM	0.05 μM
$Cr_2(SO_4)_3K_2SO_4$	0.10 μM	0.10 μM
Boric Acid	46.00 μM	46.00 μM
$MnSO_4$	9.20 μM	9.20 μM
MoO_3	1.00 μM	1.00 μM
$ZnSO_4$	0.77 μM	0.77 μM
$CuSO_4$	0.32 μM	0.32 μM
$NiSO_4$	0.10 μM	0.10 μM

[a] Used for growing *Azolla-Anabaena* association.
[b] Used for growing *Anabaena*-free *Azolla*.

1. A. Aureomycin 2 μg/ml
   B. Penicillin G 50 μg/ml
   C. Streptomycin sulfate 10 μg/ml
   D. Bacitracin 10 μg/ml plus polymixin-B-sulfate 12.5 μg/ml

2. A. Polymixin-B-sulfate 25 μg/ml
   B. Tetracycline 100 μg/ml
   C. Bacitracin 20 μg/ml
   D. Penicillin G 100 μg/ml plus streptomycin sulfate 20 μg/ml

3. A. Tetracycline 10 μg/ml, polymixin-B-sulfate 25 μg/ml, bacitracin 20 μg/ml
   B. Penicillin G 100 μg/ml, streptomycin sulfate 20 μg/ml, aureomycin 4 μg/ml
   C. Tetracycline 10 μg/ml, polymixin-B-sulfate 25 μg/ml, bacitracin 20 μg/ml
   D. Penicillin G 100 μg/ml, streptomycin sulfate 20 μg/ml, aureomycin 4 μg/ml

For experiments requiring cyanobionts directly isolated from *Azolla*, methods are available for isolating cyanobacterial packets from *Azolla* fronds in large quantities. Some of these methods involve use of enzymes to disintegrate *Azolla* tissue. However, the method described by Peters and Mayne gives very good result, without any need to use enzymic digestion.[8] This method is described below.

Azolla plants are first thoroughly washed with distilled water, after which the roots are cut and removed. The fronds are then gently crushed with a rolling pin in BG-11o medium containing PVP (polyvinyl pyrrolidone; 1%). This is then filtered through a coarse nylon mesh followed by filtration through 2 to 12 layers of cheesecloth. The filtrate is repeatedly centrifuged at low speed to yield *Anabaena azollae* in the pellet. The method yields remarkably clean cells.

Isolation and culture of the cyanobiont from *Azolla* has been tried extensively, but the success has been equivocal. The following procedure was adopted by Newton and Herman.[9]

First, 40 g (fresh weight) *Azolla* is suspended in 100 ml of *Azolla* growth medium containing 10 m*M* DTT (dithiotreitol), 500 mg pectinase, and 500 mg cullulase. This suspension is taken up in a 500-ml suction flask. The flask is evacuated and filled with Argon, to facilitate infiltration of enzymes into the *Azolla* tissue, and incubated for 16 h at 25°C. This is then diluted with equal volume of sterile distilled water and filtered through a coarse mesh into a sterile 250-ml graduated cylinder. The cyanobacterial packets are allowed to settle and are then repeatedly washed by decantation with sterile distilled water. The resulting cyanobacterial pellet is taken up in 10 ml sterile distilled water, and 1 ml from this is inoculated into 15 ml of BG-11o medium. This is then incubated under light for 2 weeks. At first, the cyanobacterial pigments are lost and the inoculum sediments to the bottom as a brown residue. Subsequently, the culture begins to turn green. Pure cultures are obtained by picking out short filaments of cyanobacteria on BG-11o agar plates using a micromanipulator and using them as inoculum.

Azolla leaf cavities contain specialized structures called hair cells, which are suggested to play a role in transfer of metabolites between the cyanobiont and *Azolla* plant. A procedure to isolate hair cells from *Azolla* cavities for experimental work has been described by Uheda.[10] *Azolla* plants, rinsed with distilled water and with the roots removed, are transferred to a solution containing 0.1% (v/v) Triton X-100 for 30 s, after which the fronds are rinsed with distilled water and vacuum infiltrated with 0.6 *M* mannitol. The fronds are then suspended in an enzyme solution containing 2% (w/v) cellulase, 0.1% (w/v) pectolyase, 1% (w/v) PVP (polyvinyl pyrrolidone; M_r 7 × 10^6), 10 m*M* DTT, and 0.6 *M* mannitol. This is incubated for 4 h at 30°C with gentle shaking. Subsequently, the enzyme solution is diluted tenfold with 0.6 *M* mannitol supplemented with 1% (w/v) PVP and subjected to repeated gentle pipetting. The slurry is then passed through a nylon mesh (pore size 410 μm) and allowed to settle. The settled plant material is resuspended in 0.6 *M* mannitol supplemented with PVP and allowed to sediment again. This procedure is repeated until most of the cyanobacterial packets are recovered. These packets are then suspended in 12.5% Percoll solution which contains 0.6 *M* mannitol (pH adjusted to 6) and allowed to sediment. Floating packets are collected, washed with 0.6 *M* mannitol, and finally resuspended in 0.6 *M* mannitol containing 10 m*M* DTT. The suspension of packets is then gently pipetted several times to yield ruptured packets. These are passed through a nylon mesh (pore size 350 μm) and centrifuged (100 × g; 5 min). The pellet is resuspended in 34% Percoll solution containing 0.6 *M* mannitol and 10 m*M* DTT, and centrifuged at 200 × g for 30 min. The supernatant contains crude hair cells. These are resuspended in 34% Percoll solution and recentrifuged. Further purification can be done by sieving through a layer of nylon mesh (pore size 54 μm). The hair cells retained by the net are suspended in 0.6 *M* mannitol, washed by centrifugation in a suitable buffer, and then used for experimentation.

CYCADS AND *GUNNERA*

Cycads and *Gunnera* plants are far larger than other cyanobacterial systems. They are generally maintained in pots, in glass houses, or taken directly from the field. They can also be generated from seeds. This yields cyanobiont-free plants which can be used for studying the infection mechanism, specificity of the cyanobiont, and metabolic changes during establishment of the symbiosis. In *Gunnera*, seedlings can be used for study of the gland development (see also Chapters 7 and 8).

The cyanobiont from these symbioses can be isolated and cultured rather easily using standard microbiological techniques. Starting material can be obtained from *Gunnera* glands or from thin slices of cycad root nodules.

To obtain cyanobiont cells from cycad root nodules for direct use, root nodules are cleaned and cut into thin slices. These are then put in HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid) — NaOH buffer (pH 7) for 1 h. Cyanobacterial filaments come out into the buffer which can be taken out with a syringe and cleaned by centrifugation. This gives clean cells, is rather easy, and provides a high yield as well. Whether such an approach is workable for the *Gunnera* cyanobiont is not known.

CULTIVATION OF CYANOBACTERIA

Various nutrient media used for culturing cyanobacteria are listed in Table 3. From these, the most suitable medium for a given cyanobacterial strain can be chosen. Temperature and light are the other factors whose optimum level for a given cyanobacterial strain should be determined. Rippka et al.[2] recommend BG-11 and its variant BG-11_0 nitrate) as good media for most cyanobacteria. For marine strains they recommend ASN-III or MN media.

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I am thankful to Dr. P. Lindblad, who showed me how to obtain large quantities of cyanobacterial cells from cycad root nodules for direct use. This is the method which I have mentioned in this chapter.

TABLE 3
Nutrient Media for Culturing Cyanobacteria

Compounds	Concentration (mg/l, except as noted)							
	Kratz and Myers[11]	Allen and Arnon[12]	ASM[13]	ASP-II[14]	BG-11$_o$[2]	ASN-III[2]	MN[2]	Chu-10[15,16]
Biotin	—	—	—	0.001	—	—	—	—
$CaCl_2$	—	74.00	36.00	370.00	36.00	500.00	18.00	55.00
$Ca(NO_3)_2 \cdot 4H_2O$[a]	25.00	—	—	—	—	—	—	40.00
Capantothenate	—	—	—	0.10	—	—	—	—
Citric acid	—	—	—	—	6.00	3.00	3.00	3.00
$CoCl_2 \cdot 6H_2O$	—	—	0.02	0.01	—	—	—	—
$Co(NO_3)_2 \cdot 6H_2O$	—	0.05	—	—	0.05	0.05	0.05	—
$Cr(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$	—	0.19	—	—	—	—	—	—
$CuCl_2 \cdot 2H_2O$	—	—	0.0002	0.003	—	—	—	—
$CuSO_4 \cdot 5H_2O$	0.079	0.079	—	—	0.079	0.079	0.079	0.079
Deionized Water	1,000 ml	1,000 ml	1,000 ml	1,000 ml	1,000 ml	1,000 ml	250 ml	1,000 ml
$FeCl_3$	—	—	0.60	2.30	—	—	—	—
FeEDTA	—	26.00	—	—	—	—	—	—
Ferric citrate	—	—	—	—	—	—	—	3.00
$Fe(SO_4)_3 \cdot 6H_2O$	4.00	—	—	—	—	—	—	—
Folic acid	—	—	—	0.002	—	—	—	—
H_3BO_3	2.86	2.86	2.50	34.30	2.86	2.86	2.86	2.86
Inositol	—	—	—	5.00	—	—	—	—
Iron(III)ammonium citrate	—	—	—	—	6.00	3.00	3.00	—
KCl	—			600.00	—	500.00	—	—
$K_2HPO_4 \cdot 3H_2O$	1,300.00	400.00	20.00	6.00	40.00	20.00	20.00	13.00
KNO_3[a]	1,000.00	2,000.00	—	—	—	—	—	—
$MgCl_2 \cdot 6H_2O$	—	—	40.00	—	—	2,000.00	—	—
$MgSO_4 \cdot 7H_2O$	250.00	250.00	50.00	5,000.00	75.00	3,500.00	—	25.00
$MnCl_2 \cdot 4H_2O$	1.81	—	1.40	4.30	1.81	1.81	1.81	1.81
$MnSO_4 \cdot 4H_2O$	—	2.00	—	—	—	—	—	—
MoO_3	0.02	0.15	—	—	—	—	—	—
Na·citrate·$2H_2O$	165.00	—	—	—	—	—	—	—
NaCl	—	230.00	—	18,000.00	—	25,000.00	—	
Na_2CO_3	—	—	—	—	20.00	20.00	20.00	20.00

TABLE 3 (continued)
Nutrient Media for Culturing Cyanobacteria

Compounds	Concentration (mg/l, except as noted)							
	Kratz and Myers[11]	Allen and Arnon[12]	ASM[13]	ASP-II[14]	BG-11$_o$[2]	ASN-III[2]	MN[2]	Chu-10[15,16]
Na_2EDTA	—	—	10.00	30.00	1.00	0.50	0.50	—
Na_2HPO_4	—	—	14.00	—	—	—	—	—
$Na_2MoO_4 \cdot 2H_2O$	—	—	—	—	0.39	0.39	0.39	0.39
$NaNO_3$[a]	—	—	170.00	50.00	—[b]	750.00	750.00	—
$Na_2SiO_3 \cdot 4H_2O$	—	—	—	150.00	—	—	—	44.00
Na_2WO_4	—	0.02	—	—	—	—	—	—
NH_4VO_3	—	0.02	—	—	—	—	—	—
Nicotinic acid	—	—	—	0.01	—	—	—	—
$NiSO_4 \cdot 6H_2O$	—	0.04	—	—	—	—	—	—
p-Aminobenzoic acid	—	—	—	0.10	—	—	—	—
pH (after autoclaving)	7—9	—	—	7.6—8.6	7.1	7.5	8.3	—
Seawater	—	—	—	—	—	—	750 ml	—
Thiamine-HCl	—	—	—	0.50	—	—	—	—
Thymine	—	—	—	30.00	—	—	—	—
$TiO(Cr_2O_4)_x \cdot 7H_2O$	—	0.01	—	—	—	—	—	—
Tris	—	—	—	1,000.00	—	—	—	—
Vitamin B_{12}	—	—	—	0.002	—	—	—	—
$ZnCl_2$	—	—	0.04	0.31	—	—	—	—
$ZnSO_4 \cdot 7H_2O$	0.222	0.222	—	—	0.222	0.222	0.222	0.222

[a] Should be omitted from the medium used for diazotrophic cyanobacteria.

[b] 1.5 g/l $NaNO_3$ is added to BG-11$_o$ for use with non-N_2-fixing cyanobacteria. NO_3-supplemented BG-11$_o$ is called BG-11.

REFERENCES

1. **Rai, A. N.**, Studies on the Nitrogen-Fixing Lichen *Peltigera aphthosa* Willd., Ph.D. thesis, Dundee University, Dundee, 1980.
2. **Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y.**, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J. Gen. Microbiol.*, 111, 1, 1979.
3. **Bunt, J. S.**, Isolation of bacteria-free cultures from hormogone producing blue-green algae, *Nature (London)*, 192, 1278, 1961.
4. **Rodgers, G. A.**, The Hepatic-Cyanophyte Symbiosis, Ph.D. thesis, Dundee University, Dundee, 1974.
5. **Enderlin, C. S. and Meeks, J. C.**, Pure cultures and reconstitution of the *Anthoceros-Nostoc* symbiotic association, *Planta*, 158, 157, 1983.
6. **Stein, J. R.**, A morphologic and genetic study of *Gonium pectorales*, *Am. J. Bot.*, 45, 665, 1958.
7. **Meeks, J. C., Enderlin, C. S., Joseph, C. M., Chapman, J. S., and Lollar, M. W. L.**, Fixation of $[^{13}N]N_2$ and transfer of fixed nitrogen in the *Anthoceros-Nostoc* symbiotic association, *Planta*, 164, 406, 1985.
8. **Peters, G. A. and Mayne, B. C.**, The *Azolla-Anabaena* relationship. I. Initial characterization of the relationship, *Plant Physiol.*, 53, 813, 1974.
9. **Newton, J. W. and Herman, A. I.**, Isolation of cyanobacteria from aquatic fern *Azolla*, *Arch. Microbiol.*, 120, 161, 1979.
10. **Uheda, E.**, Isolation of hair cells from *Azolla filiculoides* var. *japonica* leaves, *Plant Cell Physiol.*, 27, 1255, 1986.
11. **Kratz, W. A. and Myers, J.**, Nutrition and growth of several blue-green algae, *Am. J. Bot.*, 42, 282, 1955.
12. **Allen, M. B. and Arnon, D. I.**, Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm., *Plant Physiol.*, 30, 366, 1955.
13. **Gorham, P. R., McLachlan, J. S., Hammer, U. T., and Kim, W. K.**, Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Breb., *Verh. Int. Verein. Theor. Angew., Limnol.*, 15, 796, 1964.
14. **Provasoli, L., McLaughlin, J. J. A., and Droop, M. R.**, *Arch. Microbiol.*, 25, 392, 1957.
15. **Chu, S. P.**, The influence of the mineral composition of the medium on the growth of planktonic algae. I. Methods and culture media, *J. Ecol.*, 30, 284, 1942.
16. **Gerloff, G. C., Fitzgerald, G. P., and Skoog, F.**, The isolation, purification and culture of blue-green algae, *Am. J. Bot.*, 37, 216, 1950.

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